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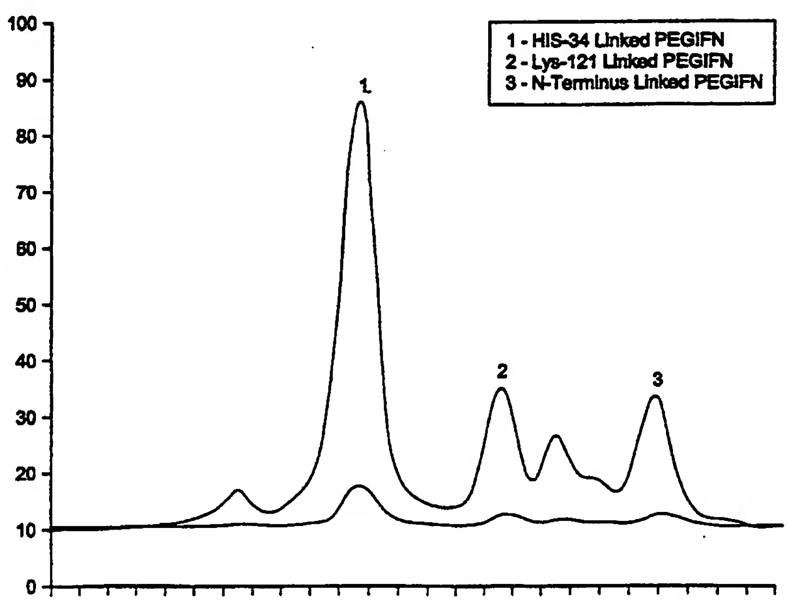

(57) Abstract

Substantially pure histidine-linked protein-polymer conjugates and processes for their preparation are disclosed. The processes include contacting a protein with an activated polymer under conditions sufficient to facilitate covalent attachment of at least a portion of the polymer strands on histidine residues of the protein and thereafter substantially separating the histidine-linked conjugates from the remaining reactants. Linkage through His-34 of alpha interferon is exemplified.

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SUBSTANTIALLY PURE HISTIDINE-LINKED PROTEIN POLYMER CONJUGATES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention is directed to substantially pure protein-polymer conjugates. In particular, the invention is directed to histidine-linked protein-polymer conjugates and methods of making the same.

2. Description of Related Art

Conjugating biologically-active proteins to polymers has been suggested to improve one or more of the properties of circulating life, water solubility or antigenicity in vivo. For example, some of the initial concepts of coupling peptides or polypeptides to polyethylene glycol (PEG) and similar water-soluble polymers are disclosed in U.S. Patent No. 4,179,337, the disclosure of which is incorporated herein by reference.

Insulin and hemoglobin were among the first therapeutic agents conjugated. These relatively large polypeptides contain several free lysine ϵ-amino attachment sites. Several polymers could be attached without significant loss of biologic activity.

For many biologically active materials, the conjugation process, however, is not without complications. The conjugation process is not specific with regard to attachment sites. Care must be taken to limit the loss of biological activity caused by the conjugation reaction. For example, if too much of the activated polymer is attached to the target protein or polypeptide, biological activity can be severely reduced or lost. Further, if the wrong linker joining the polymer to the protein is used or if an insufficient amount of polymer is attached to the target, the therapeutic value of the resultant conjugate is rather limited. Often, such conjugates do not demonstrate enough of an increase in the circulating life to compensate for the loss in bioactivity. Problems can also result when a therapeutic moiety's active site (i.e. where groups associated with bioactivity are found) becomes blocked as a result of the polymer attachment. This problem can be difficult to avoid since the polymer and protein are typically joined in solution-based reactions and the polymer conjugation process is not

specific with regard to attachment sites. Pre-blocking the active sites with materials such as pyridoxal phosphate has been suggested, but the results have been inconsistent. Lysine-depleted variants of proteins have also been suggested as a way of controlling polymer attachment. This technique, however, is often impractical since it adds significantly to the cost of the final product. The problems are particularly acute with lower molecular weight proteins and peptides. These bioactive materials often have few attachment sites not associated with bioactivity.

In another attempt to avoid the loss of bioactivity following polymer conjugation, granulocyte colony stimulating factor ("G-CSF") was conjugated to mPEG carboxymethyl-N-hydroxy-succinimidyl ester then treated with two molar hydroxylamine (pH 7.3) to remove "unstable" linkers, followed by a pH reduction to 3.5. Kinstler et al., 1996, Pharmaceutical Res. 13(7): 996-1002. No description or suggestion of attaining improved G-CSF nor guidance regarding treatment of any other protein conjugates was provided.

Interferons, hereinafter also referred to as IFN's, are a particular example of proteins which could benefit from improved polymer conjugation techniques. See, for example, U.S. Patent Nos. 4,766,106 and 4,917,888 which describe inter alia beta interferon conjugated with activated polymers including mPEG-2,4,6-trichloro-S-triazine, mPEG-N-succinimidyl glutarate or mPEG-N-succinimidyl succinate. The patentees disclose that covalent modification of the protein is done at a pH of from 5 to 9 and, when the protein is reacted through its lysine residues, covalent modification of the protein is done at a pH of from 8 to 9. Relatively high molar excesses (10, 20 and 50-fold) of the activated polymer are also used.

European Patent Application bearing publication No. 0 236 987 describes reacting alpha and gamma interferons with high molar excesses of alkyl imido ester-activated polyethylene glycols under conditions which preferably include a pH of from approximately 7 to 9. European Patent Application bearing publication No. 0 510 356 describes conjugating alpha interferon with pyridinyl carbonyl and thiocarbonyl activated PEG at a pH of from 7 to 9. There was no mention in these disclosures that amino acids other than lysine were involved in the conjugation or that it would be advantageous to do so.

WO96/11953 reports that conjugates were prepared by reacting a protein, exemplified by consensus IFN, with a polymer, at an acid pH (pH 4) using a reductive alkylation reaction for the selective attachment of polymer, e.g., PEG, to the N-terminal. WO96/11953 states that this reaction selectively prevents linkage to lysine epsilon amino groups, while favoring linkage with the N-terminal alpha amino group. WO96/11953 also describes a two-step pH treatment process wherein G-CSF is reacted with a PEG at pH 8.0, followed by reduction of pH to pH 4.0, simply as a prelude to loading the product onto a separation column. WO96/11953 does not teach or suggest the advantages of an acylation reaction to selectively attach polymers to IFN residues other than the N-terminal or lysines.

In view of the above-described disclosures, it is believed that additional improvements in interferon-polymer conjugates are desirable in order to address various shortcomings. The present invention provides additional improvements to the field and thus addresses these shortcomings.

SUMMARY OF THE INVENTION

In one aspect, the present invention includes substantially pure protein-polymer conjugates. The conjugates include a protein, such as an alpha interferon, covalently conjugated to a polymer, such as a polyethylene glycol, at a histidine (His) residue of the protein. In the case of an alpha interferon, the histidine is preferably the histidine 34. Preferably, the alpha interferon is interferon α2b and the conjugates contain about one polymer strand per alpha interferon molecule. Histidine-linked mono-polymer conjugates of other proteins, such as IL-10, are also included as part of the invention. Compositions containing the preferred mono-polymer His-linked conjugates may also contain minor amounts of other mono-PEG-protein species, if desired.

In another embodiment of the invention, methods of preparing substantially pure protein-polymer conjugates are provided. In particular, the methods are directed to preparing the protein-histidine residue linked polymer-conjugates. The methods include forming a plurality of protein-polymer conjugate species or positional isomers by reacting a protein such as alpha interferon, with a sufficient amount of suitably activated polymer under conditions sufficient to facilitate covalent attachment of

protein molecules to activated polymer strands and thereafter substantially isolating the conjugated species or positional isomers in which the His linkage between the protein and polymer is established from the remaining conjugate species. In one preferred aspect of this embodiment, the activated polymer is a benzotriazole carbonate-activated polymer. In an alternative aspect, the activated polymer is an oxycarbonyl-oxy-N-dicarboximide-activated polymer such as succinimidyl carbonate (SC-PEG). These activated polymers allow the artisan to form a reaction pool in which a substantial portion of the conjugates include the polymer strand covalently linked to a histidine residue on the alpha interferon rather than on a lysine residue or N-terminus.

Some of the conditions which allow the protein His positional isomer, such as the αIFN His34 isomer, to be formed in relatively high amounts vis à vis the other positional isomers include conducting the acylating polymer conjugation reaction within a particular pH range, i.e. preferably less than about 7 and more preferably from about 4.5 to about 6.8. This facilitates preferential covalent attachment of at least a portion of the polymer strands to histidine residue amino groups of the protein. The desired, substantially pure, protein conjugates are then preferably isolated from the remaining protein conjugates in the reaction pool using chromatography columns such as gel filtration followed by cation exchange or anion exchange followed by cation exchange.

Suitable alpha-interferons include recombinant and non-recombinant alpha-interferons isolated from mammals. The polymer portion of the conjugate is preferably a polyalkylene oxide (PAO), such as a monomethoxy polyethylene glycol (mPEG). In alternative embodiments, other substantially non-antigenic polymers can also be used. The polymers preferably have a molecular weight of from about 200 to about 35,000.

The invention also includes methods of treating various medical conditions such as alpha-interferon susceptible conditions in mammals. In this aspect, the treatment includes administering an effective amount of a composition containing the protein conjugates described herein to mammals requiring such therapy.

For purposes of the present invention, the term "positional isomer" shall be understood to generally describe a conjugate having a polymer strand attached at one of the available amino acid residues. Specific positional isomers are described herein

with reference to the amino acid residue attachment point. For example, the protein-Lys31-polymer positional isomer denotes a mono-polymer conjugate of a protein having the polymer attached at the Lys31. Other positional isomers, i.e., those conjugates having the polymer attached elsewhere on the protein would be similarly designated.

For purposes of the present invention, the term "substantially pure" shall be understood to denote the level or degree of purity of a composition containing a desired positional isomer of a protein-polymer conjugate. Depending upon the protein conjugated and the conjugate separation technique employed, compositions in accordance with the present invention will be deemed to be substantially pure if they contain a majority of the desired positional isomer. Preferably, the compositions contain at least about 60% and more preferably at least about 80% of the desired positional isomer.

Also for purposes of the present invention, "substantially separating" shall be understood to describe a part of the inventive process in which a desired positional isomer is recovered from the spectrum of positional isomers as a result of using (preferably) high performance liquid chromatography. The resulting isolates contain substantially pure isolates of the desired positional isomer and possibly minor amounts, e.g. less than 15%, of other positional isomers.

As a result of the present invention, it has been unexpectedly found that additional improvements in protein-polymer conjugate compositions are possible. For example, it is now possible to obtain substantially pure positional isomers, including those having relatively high levels of bioactivity in relatively high yields. In the case of αIFN, the preferred positional isomers, i.e. mono-polymer-His34 linked IFNα-2b conjugates, demonstrate unexpectedly high levels of bioactivity relative to not only native alpha interferon but also relative to other positional isomers. The other positional isomers, i.e., those conjugates having the polymer attached elsewhere on the interferon, such as the N-terminus or a lysine amino group, often demonstrate lower but nonetheless useful amounts of bioactivity and may be included in some inventive compositions in minor amounts.

It has also been surprisingly found that when the conjugation reaction includes certain activated polymers, such as benzotriazole carbonate (BTC) activated polymers, unexpectedly high amounts of histidine-linked positional isomers are formed.

For a better understanding of the present invention, reference is made to the following description and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a chromatogram referred to in Example 1

Figure 2 is a chromatogram referred to in Example 2.

Figure 3 is graph referred to in Example 7 illustrating the biological activity of various positional isomers in normal human serum.

DETAILED DESCRIPTION OF THE INVENTION

1. PROTEINS

For purposes of the present invention the term "protein" shall be understood to encompass not only proteins, but also polypeptides, enzymes, peptides and the like having at least one available histidine for polymer attachment. Furthermore, the proteins contemplated for use herein are not limited to those having physiological or pharmacological activities. For example, also included are enzyme conjugates which are able to catalyze reactions in organic solvents. Likewise, some inventive polymer conjugates are also useful as laboratory diagnostics. Two key features of all of the conjugates is that they are preferably linked via His residues and they maintain at least some portion of the activity associated with the unmodified protein.

Proteins, polypeptides and peptides of interest include, but are not limited to, hemoglobin, serum proteins such as blood factors including Factors VII, VIII, and IX; immunoglobulins, cytokines such as interleukins, i.e. IL-1 through IL-13, α-, β- and γ-interferons, preferably α-interferon described in more detail below, colony stimulating factors including granulocyte colony stimulating factors, platelet derived growth factors and phospholipase-activating protein (PLAP). Other proteins of general biological or therapeutic interest include insulin, plant proteins such as lectins and ricins, tumor necrosis factors and related proteins, growth factors such as transforming

growth factors, such as TGFα's or TGFβ's and epidermal growth factors, hormones, somatomedins, erythropoietin, pigmentary hormones, hypothalamic releasing factors, antidiuretic hormones, prolactin, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, tissue plasminogen activator, and the like. Immunoglobulins of interest include IgG, IgE, IgM, IgA, IgD and fragments thereof.

Some proteins such as the interleukins, interferons and colony stimulating factors also exist in non-glycosylated form, usually as a result of using recombinant techniques. The non-glycosylated versions are also among the proteins of the present invention.

Enzymes of interest include carbohydrate-specific enzymes, proteolytic enzymes, oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Without being limited to particular enzymes, examples of enzymes of interest include asparaginase, arginase, arginine deaminase, adenosine deaminase, superoxide dismutase, endotoxinases, catalases, chymotrypsin, lipases, uricases, adenosine diphosphatase, tyrosinases and bilirubin oxidase. Carbohydrate-specific enzymes of interest include glucose oxidases, glucodases, galactosidases, glucocerebrosidases, glucouronidases, etc.

Also included herein is any portion of a polypeptide demonstrating in vivo bioactivity. This includes histidine-containing amino acid sequences, antibody fragments, single chain antigen binding proteins, see, for example U.S. Patent No. 4,946,778, disclosure of which is incorporated herein by reference, binding molecules including fusions of antibodies or fragments, polyclonal antibodies, monoclonal antibodies and catalytic antibodies.

The proteins or portions thereof can be prepared or isolated by using techniques known to those of ordinary skill in the art such as tissue culture, extraction from animal sources, or by recombinant DNA methodologies. Transgenic sources of the proteins, polypeptides, amino acid sequences and the like are also contemplated. Such materials are obtained from transgenic animals, i.e., mice, pigs, cows, etc., wherein the proteins are expressed in milk, blood or tissues. Transgenic insects and baculovirus expression systems are also contemplated as sources. Moreover, mutant versions of proteins, such as mutant interferons are also within the scope of the

invention.

Other proteins of interest are allergen proteins such as ragweed, Antigen E, honeybee venom, mite allergen, and the like.

One preferred protein is alpha interferon described in more detail below. The foregoing is illustrative of the proteins which are suitable for the present invention. It is to be understood that those proteins, as defined herein, not specifically mentioned but having an available histidine group are also intended and are within the scope of the present invention.

It will also be understood by the artisan of ordinary skill that the invention includes proteins, as defined herein, which have been specifically engineered to include a histidine for use as a polymer attachment site.

In another aspect of the invention, the conjugated moiety is a non-protein-based compound such as an organically synthesized molecule which either naturally contains an amino or other suitable linking group for attaching a polymer or has been modified using standard synthetic techniques to include a histidine, tyrosine, imidazole or similar nitrogen or amine-containing group for attaching a polymer as described herein.

2. INTERFERONS

In those aspects of the invention where the protein is an interferon (IFN), it will be understood that the protein can be prepared or obtained from a variety of sources including recombinant techniques such as those using synthetic genes expressed in E. coli. See also Pestka, "Interferon α" in Human Cytokines, Blackwell Scientific Publications 1-16 (1992), the disclosure of which is incorporated herein by reference. In addition, the IFN is preferably an αIFN and can also be a mammalian source extract such as human, ruminant or bovine αIFN. One particularly preferred IFN is IFNα-2b, a recombinantly-made product of the Schering Corp., Kenilworth, NJ.

The term "interferon" or "IFN" as used herein means the family of highly homologous proteins that inhibit viral replication and cellular proliferation and modulate immune response. Human interferons are grouped into three classes based on their cellular origin and antigenicity: α-interferon (leukocytes), β-interferon (fibroblasts) and γ-interferon (B cells). Recombinant forms of each group have been

developed and are commercially available. Subtypes in each group are based on antigenic/structural characteristics. At least 24 interferon alphas (grouped into subtypes A through H) having distinct amino acid sequences have been identified by isolating and sequencing DNA encoding these peptides. See also Viscomi, 1996 Biotherapy 10:59-86, the contents of which are incorporated herein by reference. The terms "α-interferon", "alpha interferon", "interferon alpha" and "human leukocyte interferon" are used interchangeably in this application to describe members of this group. Both naturally occurring and recombinant α-interferons, including consensus interferon such as that described in U.S. Patent No. 4,897,471, the contents of which are incorporated herein by reference, may be used in the practice of the invention.

The purification of interferon alpha from human leukocytes isolated from the buffy coat fraction of whole blood is described in U.S. Patent No. 4,503,035. Human leukocyte interferon prepared in this manner contains a mixture of human leukocyte interferons having different amino acid sequences. Purified natural human α-interferons and mixtures thereof which may be used in the practice of the invention include but are not limited to Sumiferon® interferon alpha-n1 available from Sumitomo, Japan, Wellferon® interferon alpha-n1 (Ins) available from Glaxo-Wellcome Ltd., London, Great Britain, and Alferon® interferon alpha-n3 available from the Purdue Frederick Co., Norwalk, CT.

The advent of recombinant DNA technology applied to interferon production has permitted several human interferons to be successfully synthesized, thereby enabling the large-scale fermentation, production, isolation, and purification of various interferons to homogeneity. Recombinantly produced interferon retains its *in vitro* and *in vivo* antiviral and immunomodulatory activities. It is also understood that the recombinant techniques could also include a glycosylation site for addition of a carbohydrate moiety on the recombinantly-derived polypeptide.

The construction of recombinant DNA plasmids containing sequences encoding at least part of human leukocyte interferon and the expression in E. coli of a polypeptide having immunological or biological activity of human leukocyte interferon are disclosed in U.S. Patent No. 4,530,901 and European Patent No. EP 0 032 134. The construction of hybrid α-interferon genes containing combinations of different

subtype sequences (e.g., A and D, A and B, A and F) is disclosed in U.S. Patent Nos. 4,414,150, 4,456,748 and 4,678,751. Typical suitable recombinant α-interferons which may be used in the practice of the invention include but are not limited to interferon alpha-2b such as Intron® A available from Schering Corporation, Kenilworth, N.J., interferon alpha-2a such as Roferon® A available from Hoffmann-La Roche, Nutley, N.J., and Infergen® available from Amgen, Thousand Oaks, CA.

Alternate embodiments, where the foreign αIFN is not completely autologous, may be also used if desired. A key, however, is that the non-autologous αIFN has sufficient bioactivity or αIFN effect such as antiviral activity in the target mammal. Other substances including αIFN fractions or predecessor polypeptides can also be included in the conjugates of the present invention. As used herein, "α-IFN effect in mammals" means in vivo activity corresponding to that observed with αIFN's. These substances are prepared by using techniques known to those of ordinary skill in the art such as tissue culture, extraction from animal sources or by recombinant DNA methodologies. Transgenic sources of αIFN and related moieties are also contemplated. Such materials are obtained from transgenic animals, e.g. mice, pigs, cows, etc. where the αIFN protein is expressed in milk, blood, or other tissues. The method by which the αIFN is prepared for the conjugates of the present invention is not limited to those described herein. For purposes of the present invention, the αIFN's are preferred because of their biochemical and serological properties. In particular, αIFN has documented antiviral properties and diffuses more effectively into the bloodstream than other interferons.

3. NON-ANTIGENIC POLYMERS

To conjugate the protein to polymers such as poly(alkylene oxides), one of the polymer hydroxyl end-groups is converted into a reactive functional group which allows conjugation. This process is frequently referred to as "activation" and the product is called an "activated" polymer or activated poly(alkylene oxide). Other substantially non-antigenic polymers are similarly "activated" or functionalized.

In accordance with the present invention, the activated polymers are reacted with a protein such as αIFN so that the polymer attachment occurs preferably at amino groups on histidines, and, to a lesser extent, at ε-amino groups of lysines and the N-terminal amino group. Free carboxylic acid groups, suitably activated carbonyl groups, oxidized

carbohydrate moieties and mercapto groups if available on the protein can also be used as supplemental attachment sites, if desired.

In a preferred aspect of the invention, urethane (carbamate) linkages are preferably formed between a histidine amino group residue of the protein and the activated polymer. In one preferred aspect of the invention, the activated polymer is a benzotriazole carbonate-activated polymer such as those described in U.S. Patent No. 5,650,234, the disclosure of which is incorporated herein by reference. In an alternative aspect, the urethane linkage is formed using a terminal oxycarbonyl-oxy-N-dicarboximide group such as a succinimidyl carbonate group. Alternative activating groups include N-succinimide, N-phthalimide, N-glutarimide, N-tetrahydrophthalimide and N-norborene-2,3-dicarboxide. These urethane-forming groups are described in U.S. Patent No. 5,122,614, the disclosure of which is hereby incorporated by reference. When used as a part of the invention, these preferred activated polymers allow the artisan to form a plurality of protein-polymer conjugates which may or may not include the entire spectrum of positional isomers. The aggregate collection of conjugates formed in the solution-based reaction, however, will contain a significant portion of the conjugates which include the polymer strand covalently linked to a histidine residue on the target protein, i.e. alpha interferon, with lesser amounts of lysine residue linked or N-terminus linked polymer strands.

Among the substantially non-antigenic polymers, mono-activated, alkoxy-terminated polyalkylene oxides (PAO's), such as monomethoxy-terminated polyethylene glycols (mPEG's) are preferred; bis-activated polyethylene oxides (glycols) are also contemplated for purposes of cross-linking proteins or providing a means for attaching other moieties such as targeting agents for localizing the protein-polymer conjugate in a particular area such as, for example, the liver.

Suitable polymers will vary substantially by weight. Polymers having molecular number average weights ranging from about 200 to about 35,000 are usually selected for the purposes of the present invention. Molecular weights of from about 1,000 to about 25,000 are preferred and 2,000 to about 20,000 are particularly preferred.

The polymeric substances included are also preferably water-soluble at room temperature. A non-limiting list of such polymers include polyalkylene oxide homopolymers such as polyethylene glycol (PEG) or polypropylene glycols,

polyoxyethylenated polyols, copolymers thereof and block copolymers thereof, provided that the water solubility of the block copolymers is maintained. In addition to mPEG, C_{1-4} alkyl-terminated polymers are also useful.

As an alternative to PAO-based polymers, effectively non-antigenic materials such as dextran, polyvinyl pyrrolidones, polyacrylamides such as HPMA's-hydroxypropylmethacrylamides, polyvinyl alcohols, carbohydrate-based polymers, copolymers of the foregoing, and the like can be used. Those of ordinary skill in the art will realize that the foregoing list is merely illustrative and that all polymer materials having the qualities described herein are contemplated. For purposes of the present invention, "substantially or effectively non-antigenic" means all materials understood in the art as being nontoxic and not eliciting an appreciable immunogenic response in mammals.

4. **REACTION CONDITIONS**

Conjugation reactions, sometimes referred to as PEGylation reactions, are often carried out in solution without regard to where the polymer will attach to the protein. Such techniques are also usually carried out at slightly alkaline pH's, i.e. pH 7+ to about 9 for conjugating αIFNs. A key to the present invention, however, is that in certain instances, such as with αIFNs, the retained protein bioactivity can be maximized if a single polymer strand is attached to a histidine rather than a lysine or the N-terminus. In the case of αIFNs, and α IFN 2b in particular, the preferred attachment point is His34. It will be appreciated by the artisan that although various species of the αIFN may or may not have a histidine at amino acid 34, the reaction conditions will nonetheless preferably provide at least some positional isomers containing a polymer attached at an available histidine. The artisan will also appreciate that for proteins other than αIFN, the optimum histidine residue for polymer attachment will be determinable without undue experimentation.

The processes of the present invention therefore include:

1) reacting a solution containing a sufficient amount of a protein such as an alpha interferon with a sufficient amount of a suitably activated polymer, such the preferred benzotriazole carbonate-activated or oxycarbonyl-oxy-N-dicarboximide-activated polymers under conditions sufficient to facilitate covalent attachment of the protein to the activated polymer and form a plurality of protein-polymer conjugates; and

2) substantially separating the protein-polymer conjugates containing a polymer conjugated to a histidine residue of the protein from the plurality of remaining protein-polymer conjugates.

In preferred aspects when the protein is αIFN-2b, the substantially pure compositions substantially contain a polymer conjugated to the His34 of the αIFN-2b.

The reaction is conducted at a pH which is sufficient to facilitate covalent attachment of at least a portion of the polymer strands to a histidine found on the target protein. In particular, the pH is preferably be slightly acidic, i.e. less than about 7.0; more preferably, less than about 6.8 and most preferably in the range of from about 4.5 to about 6.8.

The reaction conditions for effecting conjugation further include conducting the attachment reaction with from about equi-molar to about a relatively small molar excess of the activated polymer with respect to the protein. In this regard, the process can be carried out with about 1-25-fold molar excess of polymer; preferably about 1.5-7-fold molar excess of polymer and most preferably about 1.75-5-fold molar excess of polymer. It will be understood that, depending upon the preferences of the artisan, the activated polymer may be added as a solid or in solution to the target protein. The conjugation reaction can be carried out over a relatively wide temperature range, e.g. about 0-25°C. The reaction time will also vary according to the preference of the artisan and can range from less than one hour to twenty-four hours or even longer, depending upon the activated polymer selected. Quenching of the reaction is optional. These reaction conditions provide a mixture of protein-polymer positional isomers which unexpectedly contain relatively high amounts of His-positional isomers. Preferably, each isomer contains a single polymer strand attached to the protein via an amino acid residue. In alternative embodiments, there can be more than one strand of polymer attached as a result of the conjugation process. Solutions containing these multi-stranded polymer conjugates are also useful as is or can be further processed to separate the conjugates on the basis of molecular weight to obtain mono-polymer conjugates.

5. ISOLATION OF MONO-PEG CONJUGATES

Although the inventive process produces a substantial amount of conjugates having a single polymer strand, conjugates having varying degrees of polyalkylene oxide substitution and thus molecular weight are also generated. Residual unconjugated PAO's and proteins can also be present. This mixture is typically in a reaction buffer containing one or more of phosphate, chloride and bicarbonate anions. The PAO, protein and conjugate mixture is preferably fractionated in a buffer solution containing from about 1 to about 10 mg/ml protein conjugates. Suitable fractionating solutions have a pH of from about 7.0 to about 9.0 and preferably from about 7.5 to about 8.5. The solutions preferably contain one or more buffer salts selected from KCl, NaCl, K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, $NaHCO_3$, $NaBO_4$, $(NH_4)_2CO_3$ and glycine NaOH. Sodium phosphate buffers are preferred.

Depending upon the reaction buffer, the protein-polymer conjugate containing solution may first have to undergo buffer exchange/ultrafiltration. For example, αIFN conjugate solutions can be ultra filtered across a low molecular weight cut-off (10,000 to 30,000 Dalton) membrane which will also remove most surfactants, if present, as well.

The fractionation of the conjugates into desired species based on weight is preferably carried out using an anion exchange medium. Such media are capable of selectively binding those protein-polymer conjugates having a predetermined i.e. one or more polymer strands, excess polymer and unmodified protein. This fractionation occurs since the protein molecules of various degrees of substitution will have isoelectric points which vary in a somewhat predictable fashion. For example, the isoelectric point of proteins is determined by the number of available amino groups available on the surface of the protein. These amino groups also serve as the point of attachment of polyalkylene oxide conjugates. Therefore, as the degree of substitution of polyalkylene oxide increases, the isoelectric point decreases, and the ability of the conjugate to bind to an anion exchange resin weakens. Gel filtration HPLC can also be used to remove higher molecular weight (multi-stranded) conjugates.

The use of strongly polar anion exchange resins is especially preferred for the method of the present invention. For this reason, quaternary amine coated anion exchange resins are utilized. The quaternary amine resin may be coated onto either a polymeric or

silica matrix; however, polymeric matrices are preferred. A number of tetramethylamine, or quaternary methylamine, anion exchange resins are commercially available, coated onto the support matrices. Included among the commercially available quaternary anion exchange resins suitable for use with the present invention are Q-HD available from Bio-Sepra, QA TRISACRYL® and QMA–SPHEROSIL®, quaternary amine resins coated onto a polymer matrix, manufactured by IBF of Garenne, France, for Sepracor, Inc. of Marlborough, Massachusetts; TMAE650M®, a tetramethylamino ethyl resin coated onto a polymer matrix, manufactured by EM–Separators of Gibbstown, New Jersey; QAE550C®, and SUPERQC®, each a quaternary amine resin coated onto a polymer matrix and manufactured by TosoHaas of Montgomeryville, PA. QMA Accell, manufactured by Millipore of Millford, MA and PEI resins manufactured by JT Baker of Phillipsburg, NJ, may also be used.

The anion exchange resin is packed in the column and equilibrated by conventional means. A buffer having the same pH and osmolality as the conjugated protein solution is used. The conjugate-containing solution is then adsorbed onto the column. At the completion of the loading, a gradient flow of an elution buffer with increasing salt concentrations is applied to the column to elute the desired fractions of polyalkylene oxide-conjugated protein. The fractions are of essentially uniform molecular weight and degree of substitution. Separation of the various positional isomers, however is not effected during this type of separation.

Depending upon the protein, preferred conjugate fractions have 1-4 polymer strands per protein molecule. More preferably, the fraction contains about 1-2 and, most preferably, about 1 polymer strand per protein molecule. The elution buffer preferably contains one or more salts selected from KCl, NaCl, K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, $NaHCO_3$, $NaBO_4$ and $(NH_4)_2CO_3$. These fractions are substantially free of other conjugates. Any unconjugated species can then be backwashed from the column by conventional techniques.

Techniques utilizing multiple isocratic steps of increasing concentration can also be used. Multiple isocratic elution steps of increasing concentration will result in the sequential elution of protein-polymer conjugates. The degree of polymer conjugation within each fraction will be substantially uniform. However, the degree of polymer

conjugation for each fraction will decrease with elution time. Ion exchange purification of the conjugates can also be carried out with, for example, a Q-HD Column from BioSepra, Inc. along with a dilute sodium phosphate solution. For example, samples containing PEG-IFN samples are washed with 10 mM $NaPO_4$ to remove any unreacted PAO and thereafter a step gradient elution with NaCl is used. Elution with 10 mM NaCl recovers fractions containing conjugates with greater than 3 polymer strands PAO per IFN; elution with 50 mM NaCl recovers conjugates containing 1-2 strands; elution with 150 mM NaCl recovers unmodified IFN.

The temperature range for elution is between about 4°C and about 25°C. Preferably, elution is carried out at a temperature of from about 6°C to about 22°C. The elution of the PAO-αIFN fraction is detected by UV absorbance at 254nm. Fraction collection may be achieved through simple time elution profiles. Other protein conjugates are similarly eluted.

6. SEPARATION OF POSITIONAL ISOMERS

In accordance with the method, selected positional isomers of the protein-polymer are substantially separated from the reaction mixture, preferably after the mono-polymer conjugates have been separated from the other reactants. Due to the nature of the solution-based conjugation reactions, the conjugates are a heterogenous mixture of species which contain the polymer strand(s) attached at different sites on the protein. In any solution or reaction pool containing the conjugates, it is likely that substantially the entire spectrum of positional isomers will be present. In the case of αIFN-2b, preferred conjugate-containing solutions contain conjugates in which the polymer is attached at one of three available histidine residues such as His34 and optionally at one or more of Cysl, Lys31, Lys49, Lys83, Lys121, Lys131, and Lys134 of the alpha interferon-2b. When the reaction conditions and activated polymers described herein are employed, the attachment of the polymer at a His residue on alpha interferon 2b is at least about 50% of the total reaction pool, preferably at least about 75% and most preferably at least about 85% of the conjugates in the reaction pool. For example, when BTC-activated mPEG was used to form IFNα-2b conjugates, about 90% of the conjugates formed were the IFN-His-PEG positional isomers. When SC-PEG was used, about 55% of the conjugates formed were IFN-His-PEG positional isomers. Minor amounts of the other positional isomers were

also found. It will be understood that alternative IFN's as well as other proteins will provide alternative distributions of positional isomers, depending upon the amino acid sequence of the starting material.

Applicants have determined that within the spectrum of positional isomers for any protein conjugate, biological activity of individual positional isomers will differ. While Applicants are not bound by theory, it is believed that the differences in activity for the various positional isomers are not generally predictable. In view of this determination, the methods of the present invention allow the artisan to determine which isomers provide high amounts of particular positional isomers and means for isolating the particular positional isomers from the reaction pool is highly desirable.

Separation of the desired His-positional isomers or other positional isomers from the spectrum of conjugates can be effected by methods such as ion exchange chromatography. For purposes of the present invention, ion exchange includes cation and/or anion exchange. The conjugation process leading to the formation of the various positional isomers results in the individual position isomers being formed having different charge distributions. The difference in charge distributions can then be used to resolve (recover) any desired positional isomer using ion-exchange chromatography (i.e. cation and/or anion). For example, prior to separation, the spectrum of various positional isomers resulting from the conjugation reaction are placed in a buffer solution containing from about 0.5 to about 10% conjugates by weight. The buffer solutions contain one or more buffer salts selected from the non-limiting list of KCl, NaCl, K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, $NaHCO_3$, $NaBO_4$, $(NH_4)_2CO_3$ and glycine NAOH buffers are preferred for use in the present invention. The elution conditions will, of course, depend on the needs of the artisan and the positional isomer sought.

Generally, conventional high performance liquid chromatography techniques are followed. One such apparatus for effecting the desired separation is an HPLC system comprising a Mini-S cation exchange column, available from Pharmacia Biotech. It will be apparent to those of ordinary skill that alternative apparatus and columns such as an HPLC system comprising a SP-5PW column, available form Toso Haas, will also be of use to achieve the desired separation. A non-limiting list of suitable resins for carrying out the separation includes cation or anion exchange resins such as SP-, and CM-, Q- or

DEAE Sepharose (from Pharmacia) and CM-, Q-Hyper D- from BioSepra.

As an illustrative example, a composition containing substantially pure IFNα2b-His-polymer conjugates, i.e. ≥90%, can be isolated from IFN-polymer conjugates in a reaction pool using chromatography columns such as gel filtration followed by cation exchange or anion exchange followed by cation exchange. Such techniques provide a composition containing at least about 85% IFNα2b-His34-polymer conjugates and preferably at least about 90% IFNα2b-His34-polymer conjugates. The remaining percentage of the compositions will include other positional isomers which will not appreciably detract from the therapeutic effectiveness of the desired substantially pure positional isomer. Other positional isomers of interferon or other proteins are similarly isolated. For other protein conjugates a similar separation technique is used. It will also be understood from the foregoing that linear and/or step gradient separation techniques are also useful in obtaining the conjugates corresponding to a particular peak. In addition, the conjugates associated with each peak can be isolated in this fashion, if desired. If necessary, the collected fractions can be reinjected into the chromatography apparatus with the same ratio of feed volume to column bed volume to increase the purity of the fraction collected. The substantially pure positional isomers can subjected to peptide sequencing in order to determine the amino acid residue modified.

As a further example of the techniques described above, specific IFNα-2b-polymer conjugates corresponding to particular peaks can be recovered using a cation exchange resin such as mini-S in a HPLC system. Each peak is purified on the cation exchange chromatography system using a linear gradient (A- 40mM sodium acetate, B-40mM sodium acetate, 100mM NaCl) at pH 4.7 to 5.3, wavelength 214 nanometers. Techniques using multiple isocratic steps of increasing concentration of the elution buffer, as discussed above, for the purpose of recovering the mono-polymer conjugates can also be adapted for recovery of the desired conjugates corresponding to a particular peak.

7. EFFECT OF REACTION pH UPON POSITIONAL ISOMER DISTRIBUTION

The process of the present invention takes advantage of the discovery that the site of polymer attachment on most proteins is influenced to a large extent by the pH of the reaction system. As the pH of the reaction solution is varied, the reactivity towards

specific forms of activated polymers of the various functional groups such as alpha-amines, imidazoles and epsilon amines will vary. Typically, polymer conjugation reactions are carried out at basic pHs in order to maximize attachment at lysine epsilon amino groups. For example, Zalipsky et al. Biotech. & App. Biochem, Vol 15, p.100-114; (1992) evaluated the SC-PEG reagent for PEGylation and reported that the optimal reactivity was at about pH 9.3. The method of the present invention, however, includes conducting the reaction at significantly lower pH's in order to allow a substantial portion of the activated polymer strands to attach to histidine amino groups and de-emphasize, but not eliminate, lysine and N-terminus sites for attachment.

It has also been unexpectedly determined that the relative distribution of the positional isomers is largely dependent upon the pH at which the conjugation reaction is carried out. For example, shifting the pH from basic to slightly acidic pH (about 4.5 to 6.8) favors the formation of conjugates linked at His34 on IFNα 2b, and to a lesser extent, the N-terminus (Cys1) and lysine residues. Using pH(8-10) during the conjugation reaction, on the other hand, favors the formation of lysine-related attachment sites, confirmed via cation exchange chromatography. Of course, when IFNα2b is not included, the His residue will be different. The reaction conditions nonetheless allow covalent attachment of an activated polymer to a His.

8. PHARMACOKINETIC PARAMETERS

As pointed out above, preferred compositions of the present invention do not contain a heterogeneous mixture of polymer-IFN species in which the polymer strand(s) is/are attached at different sites on the interferon molecule. Thus, the compositions have predictable in vivo pharmacokinetic and bioactivity profiles which maximize the therapeutic effect of the conjugated protein.

In the case of IFNα, some preferred compositions are substantially pure PEG-His34-IFN positional isomers. The compositions retain at least about 20%, preferably at least about 35% and most preferably at least about 50% of the unmodified protein bioactivity. It will be understood that the amount of retained activity and length of circulating life will depend upon several factors including the protein, and the number and weight of the polymer strands attached to the protein.

9. METHODS OF TREATMENT

Another aspect of the present invention provides methods of treatment for various medical conditions in mammals, preferably humans. The methods include administering an effective amount of a protein-polymer conjugate which has been prepared as described herein to a mammal in need of such treatment. The conjugates are useful for, among other things, treating conditions which are treated with the unmodified protein. For example, mammals in need of enzyme replacement therapy or blood factors can be given the substantially pure polymer conjugates containing the desired material. In the case of alpha interferon, interferon-susceptible conditions or conditions which would respond positively or favorably as these terms are known in the medical arts to interferon-based therapy.

Conditions that can be treated in accordance with the present invention are generally those that are susceptible to treatment with interferon alpha. For example, susceptible conditions include conditions which would respond positively or favorably as these terms are known in the medical arts to interferon alpha-based therapy. For purposes of the invention, conditions that can be treated with interferon alpha therapy include those conditions in which treatment with an interferon alpha shows some efficacy, but which may not be treatable with interferon alpha because the negative side effects outweigh the benefits of the treatment. For example, side effects accompanying alpha therapy have virtually ruled out treatment of Epstein Barr virus using interferon alpha. Practice of the invention results in substantially reduced or eliminated side effects as compared to conventional interferon alpha treatment.

Exemplary conditions which can be treated with interferon include but are not limited to cell proliferation disorders, in particular cancer (e.g., hairy cell leukemia, Kaposi's sarcoma, chronic myelogenous leukemia, multiple myeloma, basal cell carcinoma and malignant melanoma, ovarian cancer, cutaneous T cell lymphoma), and viral infections. Without limitation, treatment with interferon may be used to treat conditions which would benefit from inhibiting the replication of interferon-sensitive viruses. Viral infections which may be treated in accordance with the invention include hepatitis A, hepatitis B, hepatitis C, other non-A/non-B hepatitis, herpes virus, Epstein-Barr virus (EBV), cytomegalovirus (CMV), herpes simplex, human herpes virus type 6 (HHV-6)), papilloma, poxvirus, picornavirus, adenovirus, rhinovirus, human T lymphotropic virus-

type 1 and 2 (HTLV-1/-2), human rotavirus, rabies, retroviruses including human immunodeficiency virus (HIV), encephalitis and respiratory viral infections. The method of the invention can also be used to modify various immune responses.

Variants of interferon alpha are currently approved in the United States and other countries for the treatment of hairy cell leukemia, venereal warts, Kaposi's Sarcoma, and chronic non-A/non-B hepatitis: interferon alpha-2b, marketed under the trade name INTRON® A (Schering Corporation, Kenilworth N.J.), and interferon alpha-2a, marketed under the trade name Roferon® A (Hoffmann-La Roche, Nutley, N.J.), and consensus interferon marketed under the trade name Infergen™ (Amgen, Thousand Oaks, CA). Since interferon alpha-2b, among all interferons, has the broadest approval throughout the world for treating chronic hepatitis C infection, it is most preferred for use in the treatment of chronic hepatitis C in accordance with practice of the invention.

Administration of the described dosages may be every other day, but is preferably once or twice a week. Doses are usually administered over at least a 24 week period by injection.

Administration of the dose can be intravenous, subcutaneous, intramuscular, or any other acceptable systemic method. Based on the judgment of the attending clinician, the amount of drug administered and the treatment regimen used will, of course, be dependent on the age, sex and medical history of the patient being treated, the neutrophil count (e.g. the severity of the neutropenia), the severity of the specific disease condition and the tolerance of the patient to the treatment as evidenced by local toxicity and by systemic side-effects. Dosage amount and frequency may be determined during initial screenings of neutrophil count.

Conventional pharmaceutical formulations can be also prepared using the substantially pure conjugate-containing compositions of the present invention. The formulations comprise a therapeutically effective amount of the substantially pure interferon-polymer conjugate composition together with pharmaceutically acceptable carriers. For example, adjuvants, diluents, preservatives and/or solubilizers, if needed, may be used in the practice of the invention. Pharmaceutical compositions of interferon including those of the present invention may include diluents of various buffers (e.g., Tris-

HCl, acetate, phosphate) having a range of pH and ionic strength, carriers (e.g., human serum albumin), solubilizers (e.g., tween, polysorbate), and preservatives (e.g., thimerosol, benzyl alcohol). See, for example, U.S. Patent 4,496,537.

The amount of the substantially pure α-IFN polymer conjugate administered to treat the conditions described above is based on the IFN activity of the polymeric conjugate. It is an amount that is sufficient to significantly affect a positive clinical response. Although the clinical dose will cause some level of side effects in some patients, the maximal dose for mammals including humans is the highest dose that does not cause unmanageable clinically-important side effects. For purposes of the present invention, such clinically important side effects are those which would require cessation of therapy due to severe flu-like symptoms, central nervous system depression, severe gastrointestinal disorders, alopecia, severe pruritus or rash. Substantial white and/or red blood cell and/or liver enzyme abnormalities or anemia-like conditions are also dose limiting.

Naturally, the dosages of the various αIFN compositions will vary somewhat depending upon the αIFN moiety and polymer selected. In general, however, the conjugate is administered in amounts ranging from about 100,000 to about several million IU/m^2 per day, based on the mammal's condition. The range set forth above is illustrative and those skilled in the art will determine the optimal dosing of the conjugate selected based on clinical experience and the treatment indication.

The pharmaceutical compositions may be in the form of a solution, suspension, tablet, capsule, lyophilized powder or the like, prepared according to methods well known in the art. It is also contemplated that administration of such compositions will be chiefly by the parenteral route although oral or inhalation routes may also be used depending upon the needs of the artisan.

EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

EXAMPLE 1

In this example, recombinant α-interferon 2b, (rαIFN), a product of the Schering Corporation, Kenilworth, New Jersey was conjugated with activated polyethylene glycol-N-succinimidyl carbonate (SC-PEG), molecular weight 12,000 which was prepared as described in U. S. Patent No. 5,122,614. The conjugation reaction was carried out at room temperature and at a pH of about 6.5. A ratio of 2.6 grams of $SC\text{-}PEG_{12,000}$ to 1 gram of IFN was used. The SC-PEG was added as a solid and the reaction was carried out at a temperature of about 4°C. At the end of the reaction, glycine was added to quench any residual PEGylation reagent. The product from the reaction was then purified using a Q-HyperD resin at pH 8 with salt elution to remove unreacted ingredients and multi-PEGylated species. The mono-PEG-IFN recovered from the Q-HyperD resin was about 55% His-34 linked PEG-IFN, 20% N-terminus, 12% Lysine-121 with the balance being Lys 131, Lys 1134, Lys 49 and Lys 83. This material containing the several positional isomers was then dialyzed against 20 mM acetate buffer at pH 4.9 and loaded onto a column packed with SP-Sepharose High Performance equilibrate with 10 mM acetate buffer at pH 4.9 (about 4 mg of material for 4 ml of resin). The material was eluted using a sodium chloride gradient (0-500 mM) in the acetate buffer. Figure 1 shows the elution profile from the column. Peak 1 was found to be over 90% His34-linked PEG-IFN.

EXAMPLE 2

In this Example, the conjugation process of Example 1 was repeated several times. Identification of the various positional isomers, however, was determined using anion exchange followed by cation exchange.

A Mini-S cation exchange column (Pharmacia Biotech) using a HPLC was employed to determine the sites of polymer attachment and identify the individual positional isomers. Mobile phase A included 10 mM sodium acetate pH 5.3 buffer and 25% 2-propanol. Mobile phase B contained 500 mM sodium chloride dissolved in mobile phase A. The flow rate was set at 0.5 ml/min and the eluted protein was detected at 214nm. The individual PEG-IFN solutions were diluted with 10 mM sodium acetate pH 5.3, containing 2-propanol (5%) to 1 mg/ml protein concentration. Injection volumes ranged from 10 to 30 μl, depending upon the protein concentration.

The following linear gradient was used:

Time (min)	A(%)	B(%)
0	100	0
5	93	7
50	83	17
60	0	100
65	0	100
66	100	0
75	100	0

The results are provided in Table 1 below and graphically illustrated in Figure 2. Referring now to the Figure, it can be seen that Peak 3 was determined to be the major component. Furthermore, the chromatography separation resulted in recovering major peaks of differing intensity. It is to be noted, however that the individual species, i.e. positional isomers, are not fully separated from one another in this system. For example, the fraction incorporating peak 3 was determined to contain about 90% His-34 positional isomer and about 10% of the Lys-31 positional isomer. Isolation and recovery of this fraction resulted in a composition containing substantially pure αIFN2b- His-34-PEG. There is some overlap in the positional isomer elution. It can be seen, however, that peak or fraction 3 represented approximately 50% of the total PEG-interferon species.

Table 1

Area Percent Quantification of PEG-IFN Batches by Cation Exchange Chromatography

Batch	Peak 2	Peaks 3/4	Peak 5	Peak 6	Peak 7a	Peak 7b	Peak 8
1	2.6	53.2	5.3	14.2	6.5	3.4	17.2
2	1.5	54.7	3.3	12.6	6.1	3.2	18.6
3	1.6	55.3	2.4	11.9	5.5	3.2	20.1
4	1.7	55.1	2.6	11.6	5.3	3.1	20.5
5	1.7	54.3	2.7	11.8	5.6	3.2	20.7

6	1.7	54.5	2.6	11.8	5.3	2.9	21.1
7	1.9	54.2	2.3	11.6	5.2	3.2	21.5

Main Peak Assignment: Peak 2: Lys-134 linked PEG-IFN; Peak 3/4: His-34 linked PEG-IFN; Peak 6: Lys-121 linked PEG-IFN and Lys-131 linked PEG-IFN; Peak 8: Cys-1 linked PEG-IFN.

These results illustrate that a majority of the conjugates were found in peaks 3 and 4 (His-34 linked PEG-IFN). The results also show that contrary to what was expected, most of the conjugates were formed by attaching the polymer to a histidine rather than one of the lysine amino groups.

EXAMPLE 3

In this example, the various positional isomers identified in Example 2 were recovered using several cycles of a mono-S cation exchange. Each of the recovered chromatography fractions was then tested by CPE bioassay (antiviral activity). Table 2 below shows the bioactivity relative to native interferon (native = 100%).

TABLE 2

RELATIVE BIOACTIVITY (IFN)

Chromatography Fraction #	Relative Bioactivity* (%)
native IFN	100
1	17.8
2	38.4
3	50.6
4	11.2
5	17.2
6	27.6
7	11.3
8	12.8

*Bioactivity as determined by CPE Bioassay

It can be seen from Table 2 that the His34 positional isomer site (which also includes a minor amount of Lys31) possesses the highest inherent bioactivity comparative to native interferon (51%). Thus, substantially pure compositions containing only this fraction, which is mainly the His-34 positional isomer, have advantages over conjugates containing the spectrum of positional isomers.

The fractions were then characterized using an enzymatic digestion analysis scheme using Trypsin and V-8 Protease followed by a size exclusion chromatography step for clean-up. The material was subjected to protein sequence analysis wherein the presence of the pegylated amino acid residue in the interferon peptide is inferred by a vacancy in the protein sequence. The characterization work revealed that the PEG is attached at eight different sites on the α-interferon-2b molecule: Cys1, Lys31, His34, Lys49, Lys83, Lys121, Lys131 and Lys134. Details are provided below.

TABLE 3

Chromatography Fraction #	Main Site of Pegylation
1	di-PEG?
2	Lys-134
3	Lys-31 and His34
4	Lys31
5	not determined
6	Lys121 and Lys131
7	Lys49, Lys83
8	Cysteine residue/N-terminus Cys-1

EXAMPLE 4

In this example, the procedure of Example 1 was repeated using benzotriazole carbonate-activated PEG (BTC-PEG) obtained from Shearwater Polymers, Inc. (molecular weight 12,000). In particular, IFNα- 2b was reacted with BTC-PEG using a ratio of 2.6 grams of BTC per gram of IFN. The reaction was carried out at room temperature for 4 hours at a concentration of 2 mg interferon /ml before being quenched with glycine. A total of 60 mg of IFN was used. The reaction mixture was

dialyzed against a gel filtration buffer containing 100 mM sodium phosphate buffer and 150 mM sodium chloride, pH 5.0. 5 ml of the dialyzed material was loaded onto a 200 ml Superdex 200 column equilibrated with the gel filtration buffer to separate the mono-PEG species from the multistranded species.

Before conducting the characterization of the various positional isomers, the mono-PEG-IFN reaction mixture was subjected to hydroxylamine sensitivity testing to determine the percentage of the conjugates were PEGylated at histidine sites, including the IFN-His34. Hydroxylamine is known to selectively cleave PEG from IFN histidines residues. An aliquot of each of the samples (50µl) was diluted with 0.45 ml of 10 mM sodium phosphate pH 7.0 An aliquot of this protein solution (150 µl) was treated with 150 µl of 0.5 M hydroxylamine and incubated at room temperature for 60 minutes. It was determined that over 90% of the conjugates were hydroxylamine-sensitive which indicates that over 90% of the material is His-linked-PEG interferon. Further characterization of the reaction mixture verified that His34 was the only histidine residue conjugated. HPLC chromatography of the Superdex 200 pool indicated that indeed His34 was the major PEGylation site. This was further confirmed by characterization of the final product using an enzymatic digestion analysis scheme similar to the procedure described in Example 3 which indicated that His34 was the major site of PEGylation. The specific activity of this positional isomer was found to be 89 MIU/mg.

The substantially pure IFN-His34-PEG conjugates were also recovered from the BTC-PEG-IFN reaction mixture using only one cycle of cation exchange chromatography. The reaction mixture was dialyzed against 40mM sodium acetate buffer at pH 5.1. Aboput 3.2 ml of the dialyzed reaction mixture was loaded onto 4 ml of an SP-5PW column and the His34-PEG-IFN peak was eluted using a NaCl gradient (0 to 500 mM)at pH 5.1. The His34-PEG-IFN purity of the product pool was at least 94%. The di-PEG-IFN in the pool was determined to be about 3-5%.

EXAMPLES 5-6

In these examples, the process of Example 4 was repeated using BTC-PEG (molecular weight 5,000, Ex.5, and 20,000, Ex. 6, respectively). The amount of His-PEG interferon for Example 5 was determined by hydroxylamine reaction to be about 90-95% while in Example 6 it was determined to be about 91%. Isolation of the various positional isomers of PEG-IFN using gel filtration was then carried out to remove non-mono-stranded conjugates The specific activity of the $PEG_{5,000}$-conjugates was determined to be about 119 MIU/mg while the $PEG_{20,000}$-His34 positional isomer specific activity was 89 MIU/mg.

EXAMPLE 7

In this Example, the biological activity of individual positional isomers (His-34, Lys-121 and N-terminus) identified above was tested after incubation in normal human serum at 37° C for up to 72 hours and compared to nonPEGylated native interferon. The results are shown in Figure 3.

Referring now to Figure 3, it can be seen that unexpectedly only the activity of the His-34 positional isomer increased over time while the activity of the other positional isomers remained relatively constant. The native interferon, on the other hand, demonstrates a predictable drop in activity over the observation period. While Applicants are not bound by theory, the increase in activity with the His34 linked material is believed to be related to the relatively slow hydrolysis of the His-PEG bond and subsequent release of free IFN. This figure shows the unique properties of the His-PEG bond in that under certain conditions it is weaker than Lys-PEG bonds and as such its breakdown provides an extended or "slow-release" delivery mechanism.

EXAMPLES 8-9

IL-10-PEG CONJUGATES

In these examples, the protein IL-10, a non-covalent homo dimer, was conjugated to BTC-$PEG_{12,000}$ (Ex.8) or SC-$PEG_{12,000}$ (Ex. 9) at pH 6.5 in order to determine the degree of histidine-linked positional isomers in the resultant reaction pool. IL-10 has 3 available histidines.

The PEGylation procedures described above with regard to IFN were followed in order to carry out the conjugation. In particular, however, in each case a 2-3-fold molar excess of the activated polymer and gel filtration were used. Hydroxylamine sensitivity testing was done on each batch to determine the amount of His-containing positional isomers. The BTC-based conjugate was found to contain about 50% more hydroxylamine-labile conjugates than the SC-based conjugates. The specific bioactivity of the BTC-based conjugates was determined to be about 84% using the MC-9 bioassay. The SC-PEG-based conjugates were found to have a specific bioactivity of about 49%

Other embodiments of the invention will be apparent to one skilled in the art from a consideration of this specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

WHAT IS CLAIMED IS:

1. A substantially pure protein-polymer conjugate, comprising a protein covalently conjugated to a polymer at a histidine residue on said protein.
2. The protein-polymer conjugate of claim 1, wherein said polymer comprises a polyalkylene oxide.
3. The protein-polymer conjugate of claim 2, wherein said polyalkylene oxide is a polyethylene glycol.
4. The protein-polymer conjugate of claim 3, wherein said polyethylene glycol is a monomethoxy-polyethylene glycol, (mPEG).
5. The protein-polymer conjugate of claim 1, wherein said polymer has a molecular weight of from about 200 to about 35,000.
6. The protein-polymer conjugate of claim 5, wherein said polymer has a molecular weight of from about 1,000 to about 25,000.
7. The protein-polymer conjugate of claim 6, wherein said polymer has a molecular weight of from about 2,000 to about 20,000.
8. The protein-polymer conjugate of claim 1, wherein said protein is an alpha interferon or IL-10.
9. The protein-polymer conjugate of claim 8, wherein said alpha interferon is interferon alpha 2b.
10. A method of preparing protein-polymer conjugates, wherein said protein is covalently conjugated to a polymer at a histidine residue on said protein, comprising:
    a) forming a plurality of protein-polymer conjugates by reacting a protein with a sufficient amount of an activated polymer under conditions sufficient to facilitate covalent attachment of said protein to said activated polymer; and
    b) substantially separating the protein-polymer conjugates containing a polymer conjugated to a histidine of the protein from said plurality of protein-polymer conjugates.
11. The method of claim 10, wherein said protein is alpha interferon or IL-10.

12. The method of claim 10, wherein said activated polymer is benzotriazole carbonate-activated polymer.
13. The method of claim 10, wherein said activated polymer is an oxycarbonyl-oxy-N-dicarboximide-activated polymer.
14. The method of claim 13, wherein said oxycarbonyl-oxy-N-dicarboximide is succinimidyl carbonate.
15. The method of claim 10, wherein said separating is effected by gel filtration chromatography followed by ion exchange chromatography.
16. The method of claim 15, wherein said ion exchange chromatography is anion exchange chromatography.
17. The method of claim 10, wherein said separating is effected by anion exchange chromatography followed by cation exchange chromatography.
18. The method of claim 10, wherein said conditions include conducting said reacting at a pH of less than about 7.0.
19. The method of claim 18, wherein said conditions include conducting said reacting at a pH of less than about 6.8.
20. The method of claim 19, wherein said conditions include conducting said reacting at a pH of from about 4.5 to about 6.8.
21. The method of claim 11, wherein said alpha interferon is alpha interferon 2b.
22. The method of claim 10, wherein said activated polymer is present in a molar excess with respect to said protein.
23. The method of claim 10, wherein said polymer comprises a polyalkylene oxide.
24. The method of claim 23, wherein said polyalkylene oxide is a polyethylene glycol.
25. The method of claim 10, wherein said polymer has a molecular weight of from about 200 to about 35,000.
26. The method of claim 25, wherein said polymer has a molecular weight of from about 1,000 to about 25,000.
27. The method of claim 26, wherein said polymer has a molecular weight of from about 2,000 to about 20,000.
28. A method of treating a medical condition in mammals, comprising administering an effective amount of the conjugate of claim 1.

29. An interferon-polymer conjugate prepared according to the method of claim 10.

30. A pharmaceutical composition comprising the protein-polymer conjugate of claim 1.

31. The protein-polymer conjugate of claim 8, wherein at least about 50% of said polymer is conjugated to said alpha interferon at the histidine residue on said alpha interferon.

32. The protein-polymer conjugate of claim 31, wherein at least about 75% of said polymer is conjugated to said alpha interferon at the histidine residue on said alpha interferon.

33. The protein-polymer conjugate of claim 32, wherein at least about 85% of said polymer is conjugated to said alpha interferon at the histidine residue on said alpha interferon.

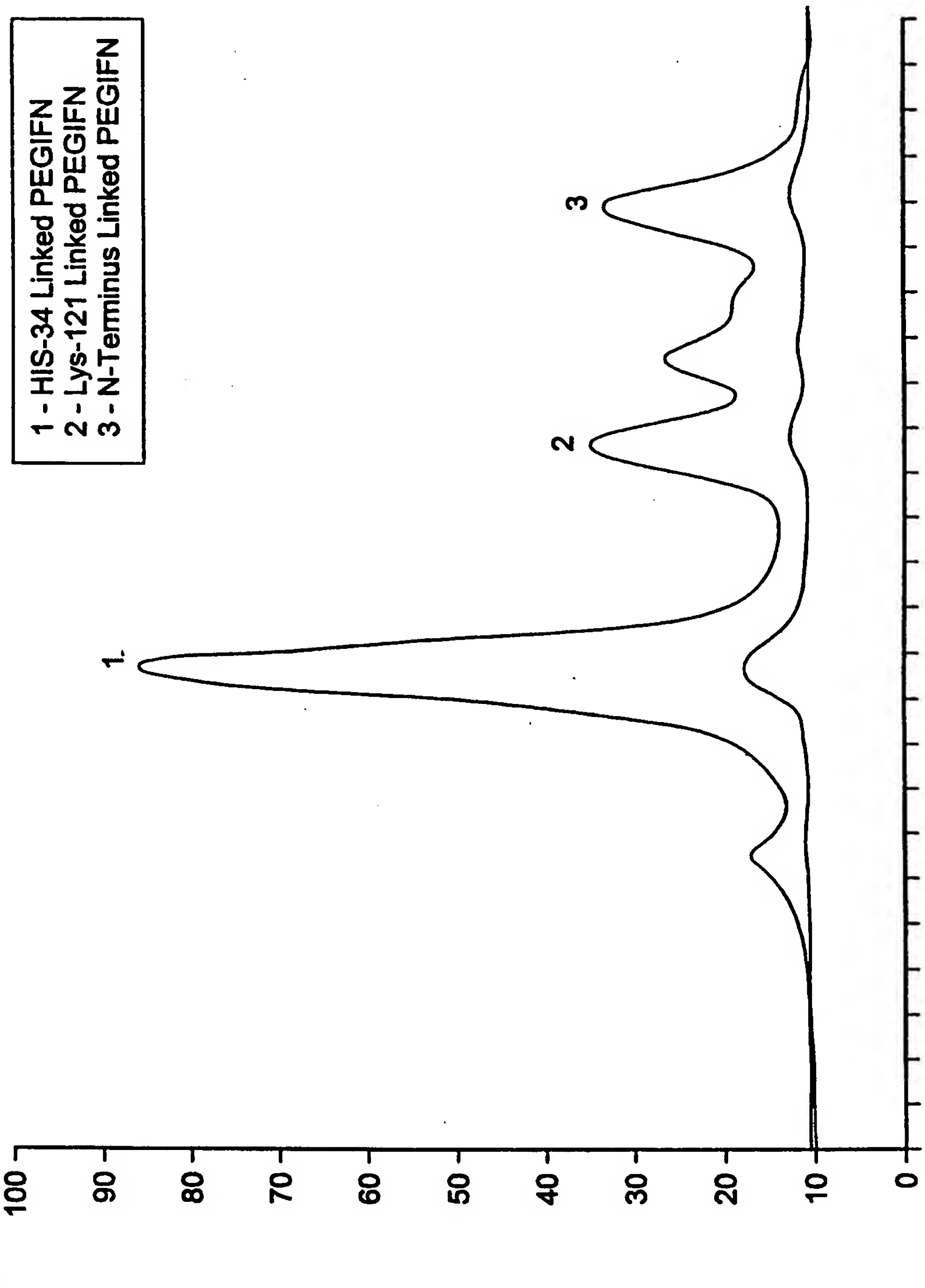

1 - HIS-34 Linked PEGIFN
2 - Lys-121 Linked PEGIFN
3 - N-Terminus Linked PEGIFN
1.
2
3
100
90
80
70
60
50
40
30
20
10
0

FIG-1

FIG-2

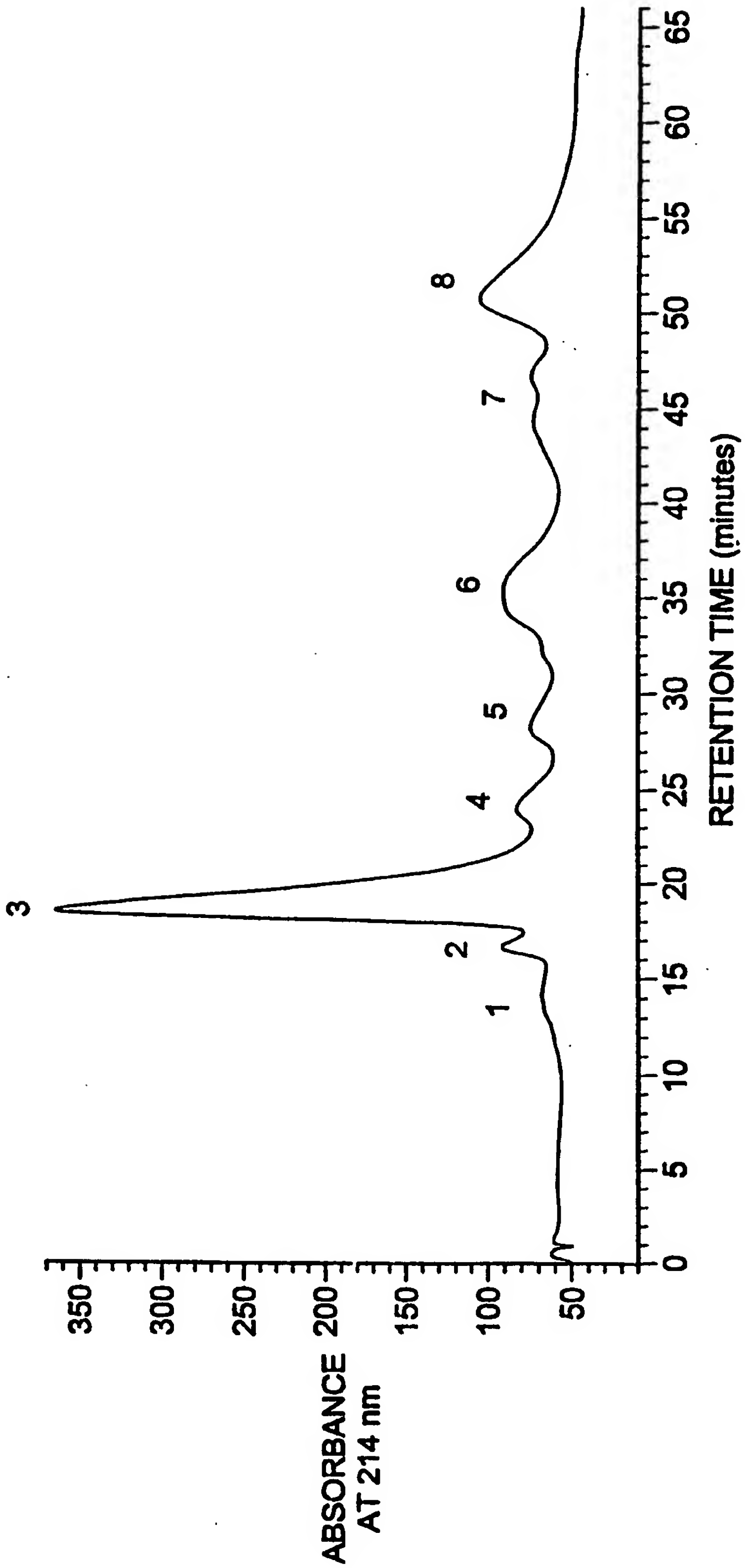
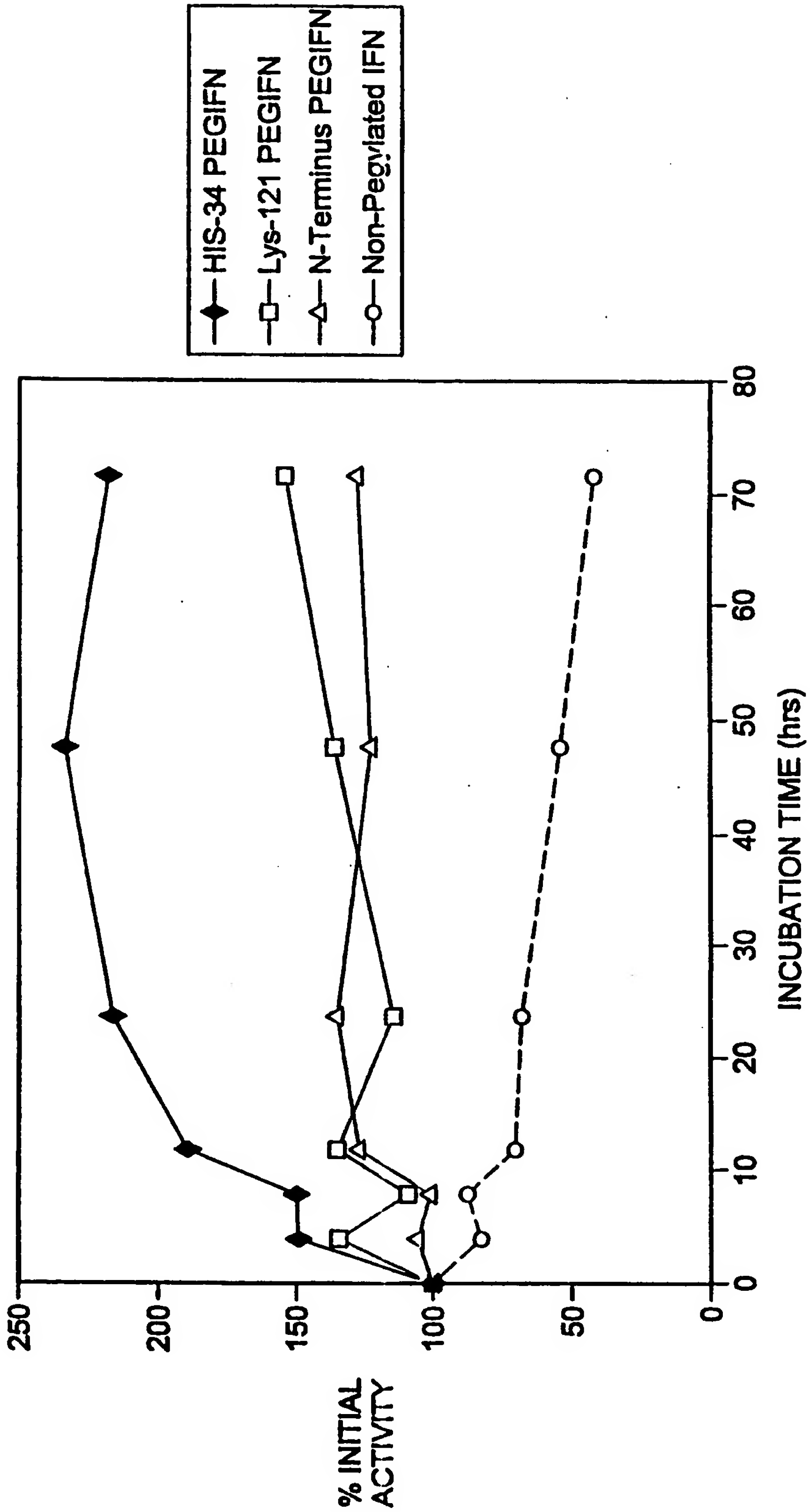

ABSORBANCE AT 214 nm
350
300
250
200
150
100
50
RETENTION TIME (minutes)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
1
2
3
4
5
6
7
8

FIG-3 BIOLOGICAL ACTIVITY IN NORMAL HUMAN SERUM @ 37 C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26676

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/02, 38/20, 38/21; C07K 1/113, 14/54, 14/56
US CL :424/85.2, 85.7; 514/2; 530/351, 409, 410
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.1, 85.2, 85.4, 85.7, 94.1, 94.3; 435/188; 514/2, 3, 12, 21; 530/300, 303, 345, 350, 351, 353, 409, 410, 411

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, DERWENT DWPI
search terms: histidine, conjugate, polyethylene glycol, interleukin, interferon, benzotriazole, succinimide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GOTOH et al. Chemical Modification of Silk Fibroin with Cyanuric Chloride-Activated Poly(ethylene glycol): Analyses of Reaction Site by ^{1}H-NMR Spectroscopy and Conformation of the Conjugates. Bioconjugate Chemistry. 1993, Volume 4, Number 6, pages 554-559, especially page 555, column 1, page 557, Scheme III.	1-7, 29, 30
A,P	US 5,711,944 A (GILBERT ET AL) 27 January 1998 (27/01/98).	1-33
A,P	US 5,738,846 A (GREENWALD ET AL) 14 April 1998 (14/04/98).	1-33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

- * Special categories of cited documents:
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Date of the actual completion of the international search	Date of mailing of the international search report
09 FEBRUARY 1999	24 FEB 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	D. Lawrence for JEFFREY E. RUSSEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BORUKHOV et al. Chemical Modification Of The Recombinant Human αA- And β-Interferons. Biochemical And Biophysical Research Communications. 28 February 1990, Volume 167, Number 1, pages 74-80.	1-33

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International Bureau

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Filed on 12 March 1998 (12.03.98)

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Published
With international search report.

(54) Title: POLY(ETHYLENE GLYCOL) DERIVATIVES WITH PROXIMAL REACTIVE GROUPS

(57) Abstract

An activated, substantially water-soluble poly(ethylene glycol) is provided having a linear or branched poly(ethylene glycol) backbone and at least one terminus linked to the backbone through a hydrolytically stable linkage, wherein the terminus is branched and has proximal reactive groups. The free reactive groups are capable of reacting with active moieties in a biologically active agent such as a protein or peptide thus forming conjugates between the activated poly(ethylene glycol) and the biologically active agent.

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POLY(ETHYLENE GLYCOL) DERIVATIVES WITH PROXIMAL REACTIVE GROUPS

Cross-Reference to Related Applications

This application is related to copending Provisional Application Serial No. 60/077,700, filed March 12, 1998, incorporated by reference in its entirety, and claims the benefit of its filing date under 35 U.S.C. §119(e).

FIELD OF THE INVENTION

This invention relates to derivatives of polyethylene glycol and related hydrophilic polymers suitable for chemical coupling to another molecule, including, for example, proteins, enzymes, small drugs, and the like.

BACKGROUND OF THE INVENTION

Chemical attachment of the hydrophilic polymer poly(ethylene glycol) ("PEG") to molecules and surfaces is of great utility in biotechnology. In its most common form PEG is a linear polymer terminated at each end with hydroxyl groups:

$$HO\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$$

This polymer can be represented in brief form as HO-PEG-OH where it is understood that the -PEG- symbol represents the following structural unit:

$$\text{-}CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}$$

In typical form n ranges from about 10 to about 2000.

PEG is commonly used as methoxy PEG-OH, or mPEG in brief, in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group that is subject to ready chemical modification.

$$CH_3O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH \quad \text{mPEG}$$

PEG is also commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. For example, the four-arm, branched PEG prepared from pentaerythritol is shown below:

$$C(CH_2\text{-}OH)_4 + n\ C_2H_4O \rightarrow C[CH_2\text{-}O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH]_4$$

The branched PEGs can be represented in general form as R(-PEG-OH)$_n$ in which R represents the central "core" molecule, such as glycerol or pentaerythritol, and n represents the number of arms.

Branched PEGs can also be prepared in which two PEG "arms" are attached to a central linking moiety having a single functional group capable of joining to other molecules; e.g., Matsushima *et al.*, (Chem. Lett., 773, 1980) have coupled two PEGs to a central cyanuric chloride moiety.

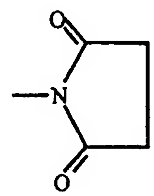
PEG is a well known polymer having the properties of solubility in water and in many organic solvents, lack of toxicity, and lack of immunogenicity. One use of PEG is to covalently attach the polymer to insoluble molecules to make the resulting PEG-molecule "conjugate" soluble. For example, it has been shown that the water-insoluble drug paclitaxel, when coupled to PEG, becomes water-soluble. Greenwald, *et al.*, *J. Org. Chem.*, 60:331-336 (1995).

In related work, U.S. Patent 4,179,337 to Davis *et al.* discloses that proteins coupled to PEG have enhanced blood circulation lifetime because of reduced rate of kidney clearance and reduced immunogenicity. These and other applications are also described in *Biomedical and Biotechnical Applications of Polyethylene Glycol Chemistry*, J. M. Harris, Ed., Plenum, New York (1992), and *Poly(ethylene glycol) Chemistry and Biological Applications*, J.M. Harris and S. Zalipsky, Eds., ACS, Washington DC (1997).

To couple PEG to a molecule such as a protein, it is often necessary to "activate" the PEG to prepare a derivative of the PEG having a functional group at the terminus. The functional group can react with certain moieties on the protein such as an amino group, thus forming a PEG-protein conjugate. Many activated derivatives of PEG have been described. An example of such an activated derivative is the succinimidyl succinate "active ester":

$$CH_3O\text{-}PEG\text{-}O_2C\text{-}CH_2CH_2\text{-}CO_2\text{-}NS$$

where NS =



Hereinafter, the succinimidyl active ester moiety will be represented as $-CO_2$-NS in chemical drawings.

The succinimidyl active ester is a useful compound because it reacts rapidly with amino groups on proteins and other molecules to form an amide linkage (-CO-NH-). For example, U.S. Patent 4,179,337 to Davis *et al.* describes coupling of this derivative to proteins (represented as PRO-NH_2):

$$mPEG\text{-}O_2CCH_2CH_2CO_2NS + PRO\text{-}NH_2 \rightarrow mPEG\text{-}O_2C\text{-}CH_2CH_2\text{-}CONH\text{-}PRO$$

Bifunctional PEGs with active groups at both ends of the linear polymer chain are also useful compounds when formation of a crosslinked insoluble network is desired. Many such bifunctional PEGs are known in the art. For example, U.S. Patent 5,162,430 to Rhee, *et al.* discloses using such bifunctional PEGs to crosslink collagen.

Reactive PEGs have also been synthesized in which several active functional groups are placed along the backbone of the polymer. For example, lysine-PEG conjugates have been prepared in the art in which a number of activated groups are placed along the backbone of the polymer. Zalipsky *et al.* *Bioconjugate Chemistry*, 4:54-62 (1993).

U.S. Patent 5,283,339 to Arnold *et al.* discloses PEG compounds capable of chelating metals. The PEG compounds have a terminal metal chelating group which has two free carboxylic acid or amino groups, typcally linked to a nitrogen atom. The PEG compounds are used to extract and precipitate proteins from solutions with the carboxylic acid or amino groups together with the nitrogen atom capable of forming ionic complexes with metal ions. However, the metal chelating groups disclosed in the patent generally are not useful in covalently coupling the PEG compounds to proteins, peptides, or small drugs bearing functional groups such as amines. The patent does not teach forming an activated PEG derivative for covalently coupling to another molecule to form a conjugate.

SUMMARY OF THE INVENTION

The invention described herein provides a water soluble polymer such as poly(ethylene glycol) or related polymers that have a branched moiety at one end of the polymer chain and two free reactive groups linked to the branched moiety for covalent attachment to another molecule. Each reactive moiety can have a tethering group, such as an alkyl chain, linking a reactive group to the branched moiety. Thus, the branched terminus allows the activated water soluble polymer of this invention to react with two molecules to form conjugates.

Because a tethering group having a desirable length can be selected in preparing an activated polymer, the two reactive groups can be held at a predetermined distance apart from each other. The two molecules conjugated to the activated polymer through the two reactive groups can also be held at a predetermined distance apart. Accordingly, an activated PEG is provided in accordance with the invention having two free reactive moieties branching out from one PEG chain at a branched moiety. The two free reactive moieties are capable of reacting with biologically active agents such as proteins, thereby linking the activated polymer to the biologically active agents.

In accordance with one embodiment of this invention, an activated water soluble polymer is provided having the formula:

$$\mathrm{POLY}-\mathrm{Y}-\mathrm{CH'}\begin{matrix}\diagup \mathrm{X} \\ \diagdown \mathrm{X'}\end{matrix}$$

wherein POLY is a water soluble, substantially non-immunogenic polymer backbone, Y is a hydrolytically stable linkage, X and X' are reactive groups capable of reacting with a moiety in another molecule such as a protein. Typically, the polymer backbone is selected from the group consisting of linear and branched poly(ethylene glycol), linear and branched poly(alkylene oxide), linear and branched poly(vinyl pyrrolidone), linear and branched poly(vinyl alcohol), linear and branched polyoxazoline, linear and branched poly(acryloylmorpholine), and derivatives thereof. Preferably, the polymer backbone is poly(ethylene glycol) or a derivative thereof. The polymer backbone POLY can have a capping group selected from the group consisting of – OH, alkyls, and –Y-CHXX' wherein Y, X and X' are as described above and can be the same or different on each terminus of the PEG.

In a preferred embodiment, X and X' are represented by -W-Z and –W'-Z' respectively, in which Z and Z' represent reactive moieties for conjugating the polymer to another molecule. W and W' represent tethering groups comprising a substantially linear chain of atoms, e.g., alkyl chains, ether chains, ester chains, amide chains, and combinations thereof. Examples of the reactive moieties include, but are not limited to, active esters, active carbonates, aldehydes, isocyanates, isothiocyanates, epoxides, alcohols, maleimides, vinylsulfones, hydrazides, dithiopyridines, and iodoacetamides.

In another embodiment of the activated polymer of this invention, the activated water soluble polymer has the formula:

$$\mathrm{R}-\{\mathrm{POLY}-\mathrm{Y}-\mathrm{CH}\begin{matrix}\diagup \mathrm{X} \\ \diagdown \mathrm{X'}\end{matrix}\}_n$$

wherein

R is a central branch core;

POLY is a water soluble substantially non-immunogenic polymer;

Y is a hydrolytically stable linkage;

n is from 2 to 200;

X and X' are reactive groups capable of reacting with a moiety in another molecule such as a protein.

Many central branch core molecules for preparing branched or dendritic PEGs are known and can all be used for R. Typically, R can be a moiety derived from lysine, glycerol, pentaerythritol, or sorbitol. Suitable polymer backbones include, but are not limited to, linear and branched poly(ethylene glycol), linear and branched poly(alkylene oxide), linear and branched poly(vinyl pyrrolidone), linear and branched poly(vinyl alcohol), linear and branched polyoxazoline, linear and branched poly(acryloylmorpholine), and derivatives thereof. Preferably, is poly(ethylene glycol) or a derivative thereof is used as the polymer backbone.

The reactive groups X and X' can be reactive moieties directly linked to the branching moiety –CH. Preferably, X and X' have a tethering group and are represented by -W-Z and –W'-Z' respectively, in which Z and Z' represent reactive groups for conjugating the polymer to another molecule. W and W' represent tethering groups comprising a substantially linear chain of atoms, e.g., alkyl chains, ether chains, ester chains, amide chains, and combination thereof. Examples of the reactive groups include, but are not limited to, active esters, active carbonates, aldehydes, isocyanates, isothiocyanates, epoxides, alcohols, maleimides, vinylsulfones, hydrazides, dithiopyridines, and iodoacetamides.

The activated water soluble polymer can be covalently linked to a biologically active agent to form a conjugate. A suitable biologically active agent can be any of those having a moiety capable of reacting with at least one of the two reactive groups in the terminus of the activated polymer. The biologically active agent can have two such moieties and each of them can be linked to one of the two reactive groups. Alternatively, the conjugate can have two biologically active agents each being linked to one of the two reactive moieties of the activated polymer. Because activated polymers having different tethering groups can be prepared in accordance with this invention, an activated polymer can be provided

in which the two reactive groups in a terminus of the activated polymer are a desirable distance from each other. When such an activated polymer is conjugated to two biologically active agent molecules, the two molecules can be held at a desired distance apart.

Accordingly, the activated PEG can be used with greater versatility as compared to other PEG derivatives heretofore known in the art to form various conjugates with molecules such as proteins or peptides. Since PEG molecules conjugated to another molecule can impart water solubility and reduced immunogenicity to the other molecule, , the activated PEG derivatives of this invention allows greater control and precision in modifying such characteristics in a conjugate.

Thus, an activated water soluble polymer having proximal reactive groups is provided. The polymer backbone has at least one terminus having two reactive groups. The terminus has a branching moiety and two free reactive moieties linked to the branching moiety. The branching moiety is in turn linked to the polymer backbone through a stable linkage.

DETAILED DESCRIPTION OF THE INVENTION

The terms "group," "functional group," "moiety," "active moiety," "reactive site," reactive group" and "reactive moiety" are used in the art and herein to refer to distinct, definable portions or units of a molecule. The terms are somewhat synonymous in the chemical arts and are used herein to indicate that the portions of molecules that perform some function or activity and are reactive with other portions of molecules.

The term "linkage" is used herein to refer to groups or bonds that normally are formed as the result of a chemical reaction and typically are covalent linkages. Hydrolytically stable linkages means that the linkages are substantially stable in water and do not react with water at useful pHs, e.g., under physiological conditions for an extended period of time, preferably indefinitely.

The term “biologically active agent” when used herein means any substance which can affect any physical or biochemical properties of a biological organism, including but not limited to viruses, bacteria, fungi, plants, animals, and humans. In particular, as used herein, biologically active agent includes any

substance intended for the diagnosis, cure, mitigation, treatment, or prevention of disease in humans or other animals, or to otherwise enhance physical or mental well being of humans or animals. Examples of biologically active agents include, but are not limited to, organic and inorganic compounds, proteins, peptides, lipids, polysaccharides, nucleotides, DNAs, RNAs, other polymers, and derivatives thereof. Examples of biologically active agents include antibiotics, fungicides, anti-viral agents, anti-inflammatory agents, anti-tumor agents, cardiovascular agents, anti-anxiety agents, hormones, growth factors, steroidal agents, and the like. Other examples include, microorganisms such as bacteria and yeast cells, viral particles, plant or animal or human cells, and the like.

The polymer backbone is a water soluble substantially non-immunogenic polymer, and is preferably poly(ethylene glycol) (PEG). However, it should be understood that other related polymers are also suitable for use in the practice of this invention and that the use of the term PEG or poly(ethylene glycol) is intended to be inclusive and not exclusive in this respect.

Poly(ethylene glycol) or PEG is useful in biological applications because it has properties that are highly desirable and is generally approved for biological or biotechnical applications. PEG typically is clear, colorless, odorless, soluble in water, stable to heat, inert to many chemical agents, does not hydrolyze or deteriorate, and is generally nontoxic. Poly(ethylene glycol) is considered to be biocompatible, which is to say that PEG is capable of coexistence with living tissues or organisms without causing harm. More specifically, PEG is non-immunogenic, which is to say that PEG does not tend to produce an immune response in the body. When attached to a molecule having some desirable function in the body, such as a biologically active agent, to form a conjugate, the PEG tends to mask the agent and can reduce or eliminate any immune response so that an organism can tolerate the presence of the agent. Accordingly, the conjugate is substantially non-toxic. PEG conjugates tend not to produce a substantial immune response or cause clotting or other undesirable effects. PEG having the formula $-CH_2CH_2-(CH_2CH_2O)_n-CH_2CH_2-$, where n is from about 8 to about 4000, is one useful polymer in the practice of the invention. Preferably PEG having a molecular weight of from about 200 to about 100,000 Da is used as polymer backbone.

The polymer backbone can be linear or branched. Branched polymer backbones are generally known in the art. Typically, a branched polymer has a central branch core moiety and a plurality of linear polymer chains linked to the central branch core. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, pentaerythritol and sorbitol. For example, the four-arm, branched PEG prepared from pentaerythritol is shown below:

$$C(CH_2\text{-}OH)_4 + n\ C_2H_4O \rightarrow C[CH_20\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH]_4$$

The central branch moiety can also be derived from several amino acids, e.g., lysine.

The branched polyethylene glycols can be represented in general form as R(-PEG-OH)$_n$ in which R represents the core moiety, such as glycerol or pentaerythritol, and n represents the number of arms. Suitable branched PEGs can be prepared in accordance with International Publication No. WO 96/21469, entitled *Multi-Armed, Monofunctional, and Hydrolytically Stable Derivatives of Poly(Ethylene Glycol) and Related Polymers For Modification of Surfaces and Molecules*, which was filed January 11, 1996, the contents of which are incorporated herein in their entirety by reference. These branched PEGs can then be modified in accordance with the teachings herein.

Many other water soluble substantially non-immunogenic polymers than PEG are also suitable for the present invention. These other polymers can be either in linear form or branched form, and include, but are not limited to, other poly(alkylene oxides) such as poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like; poly(vinyl alcohol) ("PVA") and the like. The polymers can be homopolymers or random or block copolymers and terpolymers based on the monomers of the above polymers, straight chain or branched.

Specific examples of suitable additional polymers include, but are not limited to, difunctional poly(acryloylmorpholine) ("PAcM"), and poly(vinylpyrrolidone)("PVP"). PVP and poly(oxazoline) are well known polymers in the art and their preparation should be readily apparent to the skilled

artisan. PAcM and its synthesis and use are described in U.S. Patent Nos. 5,629,384 and 5,631,322, the contents of which are incorporated herein by reference in their entirety.

Although the molecular weight of each chain of the polymer backbone can vary, it is typically in the range of from about 100 to about 100,000, preferably from about 6,000 to about 80,000.

Those of ordinary skill in the art will recognize that the foregoing list for substantially water soluble non-immunogenic polymer backbone is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated.

The activated polymer of this invention also has proximal reactive groups linked to at least one arm of the polymer backbone. As will be apparent, the term "proximal" is used herein to mean that the terminus has two free reactive moieties capable of reacting with two other moieties in another molecule or two other molecules, which can be the same or different.

The terminus typically has a branching moiety covalently linked to a polymer chain of the polymer backbone through a hydrolytically stable linkage. Typically, there are two free reactive groups branching out from the branching moiety. The term "free" as used herein means that each of the two free reactive groups has two ends, one of which is covalently linked to the branching moiety and the other end is not linked to any other moiety or group through covalent linkage, and (available for reaction with another moiety or group, e.g., of another molecule).

Typically the branching moiety is a stable, non-reactive, and inert moiety that is covalently linked to a polymer chain and to the two reactive groups. The branching moiety should not form a hydrogen bond or ionic bond with metal ions or moieties or molecules. It is believed that the ability to form strong hydrogen bonds or ionic bonds would interfere with the branching moiety's function. The branching atom, i.e., the atom the two free reactive groups are linked to is not a nitrogen atom (N), but is typically a carbon atom (C).

At least one of the two free reactive groups may comprise two portions: a reactive moiety at the free end and a tethering group linking the reactive moiety to the branching moiety. The reactive moiety is a moiety capable of

reacting with a moiety in another molecule, e.g., a biologically active agent such as proteins, peptides, etc. Examples of suitable reactive moieties include, but are not limited to, active esters, active carbonates, aldehydes, isocyanates, isothiocyanates, epoxides, alcohols, maleimides, vinylsulfones, hydrazides, dithiopyridines, N-succinimidyl, and iodoacetamides. The selection of a free reactive moiety is determined by the moiety in another molecule to which the free reactive moiety is to react. For example, when the moiety in another molecule is a thiol moiety, then a vinyl sulfone moiety is preferred for the free reactive moiety of the activated polymer. On the other hand, an N-succinimidyl moiety is preferred to react to an amino moiety in a biologically active agent.

The tethering group can have a predetermined length such that the reactive moiety linked to it is at a predetermined distance away from the branching moiety, and consequently, a predetermined distance from the other reactive moiety of the terminus. Typically, the tethering group is non-reactive and is a substantially linear chain of atoms, e.g., alkyl chains, ether chains, ester chains, amide chains, and combinations thereof.

Thus, in a preferred embodiment, the activated polymer of this invention can be represented by formula I:

$$\mathrm{POLY} - \mathrm{Y} - \mathrm{CH'}\begin{matrix}\diagup \mathrm{X} \\ \diagdown \mathrm{X'}\end{matrix}$$

or formula II:

$$\mathrm{R}\text{-}\{\mathrm{POLY}\text{-}\ \mathrm{Y} - \mathrm{CH}\begin{matrix}\diagup \mathrm{X} \\ \diagdown \mathrm{X'}\end{matrix}\}_n$$

In the above formula, POLY is a linear polymer chain of a water soluble substantially non-immunogenic polymer backbone, preferably is poly(ethylene glycol) or a derivative thereof. In the activated polymer represented

by formula I, the polymer backbone has only one polymer chain. Y is a hydrolytically stable linkage, which can comprise an atom or a group such as -O-, -S- and -CO-NH-. It will be apparent to skilled artisan that many other hydrolytically stable linkages can also be employed in this embodiment.

X and X' are free reactive groups, which can be same or different, each having a reactive moiety capable of reacting with a moiety in another molecule such as a protein. In the activated polymer as represented by formula I, the polymer backbone POLY can have a capping group at the end opposite to the terminus having proximal reactive groups. The capping group can be, for example, – OH, various alkyl, and can also contain proximal reactive groups –Y-CHXX' wherein Y, X and X' are as described above. Accordingly, the activated polymer can have two terminals with proximal reactive groups, one on each end of the polymer backbone.

In formula II, R is the central core as described above. POLY is a polymer chain of the water soluble substantially non-immunogenic polymer backbone. Y is a hydrolytically stable linkage. n is from 2 to 200 representing the number of polymer chains or arms in the polymer backbone, as described above.

As will be apparent, the branching moiety as described above is CH in this embodiment of the activated polymer. Typically it does not become charged in normal conditions, and does not form an ionic bond with a metal ion.

In a preferred embodiment, X and X' can have a tethering group in addition to a reactive moiety and can be represented by -W-Z and –W'-Z' respectively, in which Z and Z' represent free reactive moieties for conjugating the polymer to another molecule. W and W' represent tethering groups. Z and Z' can be different or same reactive moieties.

Some examples of preferred embodiments of the activated polymers of this invention are provided as follows:

$CH_3O\text{-}PEG\text{-}CO\text{-}NH\text{-}CH(CH_2\text{-}OCO_2\text{-}NS)_2$

$CH_3O\text{-}PEG\text{-}CO\text{-}NH\text{-}CH(CH_2\text{-}O_2C\text{-}CH_2CH_2\text{-}CO_2\text{-}NS)_2$

$CH_3O\text{-}PEG\text{-}CO\text{-}NH\text{-}CH(CH_2\text{-}CO_2\text{-}NS)_2$

$CH_3O\text{-}PEG\text{-}O\text{-}CH(CH_2\text{-}OCO_2\text{-}NS)_2$

$CH_3O\text{-}PEG\text{-}O\text{-}CH(CH_2\text{-}O_2C\text{-}CH_2CH_2\text{-}CO_2\text{-}NS)_2$

$(OHC\text{-}CH_2CH_2\text{-}O\text{-}CH_2)_2\text{-}CH\text{-}NHCO\text{-}PEG\text{-}CONH\text{-}CH(OCH_2CH_2\text{-}CHO)_2$

PEG-[CONH-CH(OCH_2CH_2-CHO)$_2$]$_4$

CH_3O-PEG-CO-NH-CH(CO_2-NS)($CH_2CH_2CO_2$-NS)

In these examples, -NS represents the N-succinimidyl moiety.

In accordance with another aspect of this invention, a method for preparing the activated water soluble polymer of this invention is also provided.

Typically, in the first step, there is a first intermediate polymer provided having a polymer backbone and a reactive end group covalently linked to of the polymer backbone.

In addition, a compound having three reactive groups linked to a branching moiety is provided. This compound typically has a branching moiety forming a central core and three free groups branching out from the central core. When the three free groups are linked to the same atom in the branching moiety, the atom is not a nitrogen atom. One of the three free groups can react with the reactive end group of the first intermediate polymer to form a hydrolytically stable linkage. The other two free groups can be ultimately converted into the two free reactive groups on the terminus of the activated polymer of this invention. Examples of these compounds include, $H_2NCH(CH_2\text{-}OH)_2$, NaO-CH(CH_2 -O-Bz)$_2$, $H_2N\text{-}CH(CH_2CO_2H)_2$, and the like. As will be apparent, in these examples, the branching moiety is CH. The H_2N- and NaO- moieties can be used to link the compound to the first intermediate polymer to form a hydrolytically stable linkage, while the hydroxyl groups, carboxylic acid groups, and -CH_2-O-Bz groups can be ultimately converted into free reactive moieties of the activated polymer of this invention.

Thus, in the second step of the method, the compound having three reactive groups is reacted with the first intermediate polymer to form a second intermediate polymer which includes a hydrolytically stable linkage linking the first intermediate polymer and the compound having three reactive groups, thus leaving only two free groups at the terminus of the polymer chain.

In the third step, the two free groups of the compound are converted into two free reactive moieties linked to the branching moiety. A number of methods known in the art can be employed in the conversion. For example, the free groups can be reacted to a compound which can impart a free reactive moiety. Alternatively, the two free groups in the second intermediate polymer can be

oxidized or reduced or substituted to form two new free reactive moieties. Such methods will be apparent to skilled artisan in view of the Examples given below.

In accordance with yet another aspect of this invention, a conjugate is provided formed by covalently linking the activated water soluble polymer of this invention to another molecule, e.g., a biologically active agent. Typically, a suitable biologically active agent can be any biologically active agent having a moiety capable of reacting with at least one of the two proximal reactive groups in the terminus of the activated polymer.

The biologically active agent can have two such moieties and each of them can be linked to one of the two reactive groups. Alternatively, the conjugate can have two biologically active agents each being linked to one of the two reactive moieties of the activated polymer. For example, the reactive moieties in the activated polymer can be vinyl sulfone moieties, which can react with a thiol moiety. If a protein has only one thiol moiety, then two of such protein molecules can be linked to the activated polymer through the two vinyl sulfone moieties. When a protein has two thiol moieties, the reaction between the protein and the activated polymer can be controlled such that each activated polymer molecule is conjugated to two protein molecules. Alternatively, the reaction can also be controlled such that the two vinyl sulfone moieties of an activated polymer are reacted with two thiol moieties on the same protein molecule.

Other moieties in biologically active agents useful for reacting with the free reactive moieties of the bivalent terminus of an activated polymer of this invention include, e.g., amino groups, carboxylic acid groups, etc. It will be apparent for skilled artisan once apprised of the present invention to select appropriate free reactive moieties in an activated polymer for reaction with a given moiety in a biologically active agent. For example, if conjugation is through reaction with an amino group in a biologically active agent, moieties such as -CO_2-NS or aldehyde is preferably used as a free reactive moiety in the activated polymer for conjugation.

Because activated polymers having different tethering groups can be prepared in accordance with this invention, an activated polymer can be provided in which the two reactive groups in a bivalent terminus of the activated polymer are in a desirable distance from each other. When such an activated

polymer is conjugated to two biologically active agent molecules, the two molecules can be held at a desired distance apart.

The following examples are given to illustrate the invention, but should not be considered in limitation of the invention:

Example 1. Synthesis of $mPEG_{20K}$-$OCH_2CH_2CONHCH$ $(CH_2O_2CCH_2$ $CH_2CO_2NS)_2$

(NS=N-succinimidyl)

Example 2. Synthesis of $mPEG_{20K}$-$OCH(CH_2$-$SO_2CH{=}CH_2)_2$

Example 3. Synthesis of $mPEG_{5K}$-O_2CNH-$CH(CH_2CO_2NS)_2$

Example 4. Synthesis of $mPEG_{5k}$-O-$CH_2CH_2CH(CO_2H)_2$

EXAMPLE 1

Reactions:

1. $mPEG_{20K}$-$OCH_2CH_2CO_2NS$ + $H_2NCH(CH_2$-$OH)_2$

$mPEG_{20K}$-$OCH_2CH_2CONHCH(CH_2OH)_2$ + NHS

NS = N-succinimidyl;

NHS = N-hydroxysuccinimide

2. $mPEG_{20K}$-$OCH_2CH_2CONHCH(CH_2$-$OH)_2$ + 2SA

$mPEG_{20K}$-$OCH_2CH_2CONHCH(CH_2$-$O_2CCH_2CH_2CO_2H)_2$

SA = succinic anhydride

3. $mPEG_{20K}$-$OCH_2CH_2CONHCH(CH_2$-$O_2CCH_2CH_2CO_2H)_2$ + NHS + DCC

$mPEG_{20K}$-$OCH_2CH_2CONHCH(CH_2$-$O_2CCH_2CH_2CONS)_2$

DCC = dicyclohexylcarbodiimide

Preparations:

1. $mPEG_{20K}$-$OCH_2CH_2CONHCH(CH_2OH)_2$

A solution of $mPEG_{20K}$-$OCH_2CH_2CO_2NS$ (mSPA 20K, 20 g, 0.001 moles)), $H_2NCH(CH_2$-$OH)_2$ (serinol, , 0.14 g, 0.00154 moles), and triethylamine

(0.3 ml) in acetonitrile (100 ml) was stirred under nitrogen overnight and the solvent removed by distillation. The product was chromatographed on DEAE sepharose eluted with water and the eluate was saturated with NaCl and extracted with chloroform. The resulting chloroform phase was dried over magnesium sulfate, filtered, and the filtrate evaporated to dryness under vacuum to yield 20 g of product as a white solid showing a single peak with gel permeation chromatography (Ultrahydrogel 250, pH 7.2 buffer).

2. $mPEG_{20K}-OCH_2CH_2CONHCH(CH_2-O_2CCH_2CH_2CO_2H)_2$

A solution of the product from (1) (20 g, .002 moles) and butylated hydroxytoluene (BHT) (0.02g) in 220 ml of chloroform was subjected to distillation until about 150 ml of solvent had distilled. Succinic anhydride (2.0 g, 0.02 moles), pyridine (1.62 ml, 0.02 moles), and 40 ml of toluene were added and the resulting mixture heated at 84 C for 20 h under nitrogen. The product was precipitated with 850 ml of ether and collected by filtration. After drying, the product was dissolved in 200 ml of water, 20 g of NaCl added, and the pH adjusted to 3 with aqueous phosphoric acid. The product was extracted with chloroform (200 + 150 + 100 ml) and the combined extracts dried over magnesiun sulfate. Evaporation of the dried solution yielded the product as a white solid (16 g). The molecular weight was determined to be 20,940 Da by potentiometric titration.

3. $mPEG_{20K}-OCH_2CH_2CONHCH(CH_2-O_2CCH_2CH_2CONS)_2$

A solution of the product from (2) (15 g, 0.0015 moles), N-hydroxysuccinimide (0.21 g, 0.00179 moles), dicyclohexylcarbodiimide, 0.37 g, 0.00177 moles) in methylene chloride (100 ml) was stirred at room temperature under nitrogen overnight. The suspension was filtered, product precipitated twice from methylene chloride by addition of ether (850 ml) and collected by filtration to obtain a white solid (13.0 g) which had 97.7% substitution by proton nmr. The proton nmr displayed a broad multiplet at 3.50 ppm (PEG backbone methylene groups), a singlet at 3.23 ppm PEG methyl), a singlet at 2.80 ppm (NS methylenes), and multiplets at 2.68 and 2.95 ppm (succinate methylenes).

EXAMPLE 2

Reactions:

1. $HO\text{-}CH(CH_2\text{-}O\text{-}Bz)_2$ + NaH (toluene) $NaO\text{-}CH(CH_2\ OBz)_2$

   Bz = Benzyl

2. $NaO\text{-}CH(CH_2\ \text{-}O\text{-}Bz)_2 + mPEG_{20K}\text{-}O\text{-}Ms$ $mPEG_{20K}O\text{-}CH(CH_2OBz)_2$

   Ms = mesylate

3. $mPEG_{20K}O\text{-}CH(CH_2OBz)_2 + HCO_2H/MeOH/H_2O/Pd/C$

   $mPEG_{20K}O\text{-}CH(CH_2OH)_2$

4. $mPEG_{20K}O\text{-}CH(CH_2OH)_2 + MsCl/Et_3N$ $mPEG_{20K}O\text{-}CH(CH_2OMs)_2$

5. $mPEG_{20K}O\text{-}CH(CH_2OMs)_2 + HSCH_2CH_2OH$
   $mPEG_{20K}O\text{-}CH(CH_2SCH_2CH_2OH)_2$

6. $mPEG_{20K}O\text{-}CH(CH_2SCH_2CH_2OH)_2 + H_2WO_4$
   $mPEG_{20K}O\text{-}CH(CH_2SO_2CH_2CH_2OH)_2$

7. $mPEG_{20K}O\text{-}CH(CH_2SO_2CH_2CH_2OH)_2 + MsCl/ET_3N$
   $mPEG_{20K}O\text{-}CH(CH_2SO_2CH{=}CH_2)_2$

Preparations:

1. $mPEG_{20K}O\text{-}CH(CH_2OBz)_2$

A solution of 18 g (0,0641 moles) of 1,3-dibenzyloxy-2-propanol in 80 ml of toluene was distilled until 15 ml of toluene was removed. The azeotropically dried solution was then added to a suspension of 2.56 g (0.064 moles) of NaH in 80 ml of toluene and the resulting mixture stirred while heating to 37-40 C before filtering. The filtrate was then added to a solution of azeotropically-dried $mPEG_{20K}$ mesylate in about 350 ml of toluene and the resulting mixture was heated for 20 h at 125 C under N_2. The product was precipitated with cold ether, wash on the filter with hexane, and dried under vacuum to yield 70.4 g of white solid shown to be pure by proton nmr.

2. $mPEG_{20K}O\text{-}CH(CH_2OH)_2$

To a solution of 15 g (0.00075 moles) of the product from (1) in 9.2 ml of formic acid and 0.8 ml of water was added 2.0 g of Pd/C (10%) and the mixture was stirred for 2 h under nitrogen. The mixture was then filtered and the pH of the filtrate adjusted to 7.2. The reuslting solution was extracted with CH_2Cl_2 and the extract dried over $MgSO_4$. Evaporation of the solution yielded 12.9 g of product which displayed no benzyl groups in the proton nmr.

3. $mPEG_{20K}O\text{-}CH(CH_2OMs)_2$

To an azeotropically-dried solution of the product from (2) (8.0 g, 0.000889 moles) in toluene (100 ml) containing 0.008 g of BHT was added a solution of mesyl chloride (0.090 ml, 0.00116 moles) and triethylamine (0.210 ml, 0.0011 moles) in 10 ml of ET_3N and the resulting solution was stirred overnight at room temperature under nitrogen. Ethanol (1 ml) was added and 50 ml of the solvent was removed by distillation before adding 500 ml of ether to precipitate the product. The product was collected by filtration and dried under vacuum to yield 7.6 g of the mesylate derivative shown by nmr to be 100% substituted.

4. $mPEG_{20K}O\text{-}CH(CH_2SCH_2CH_2OH)_2$

A solution of the product of (3), (7.0 g, 0.00035 moles), mercaptoethanol (0.56, 0.0080 moles) ml, NaOH (0.22 g), in toluene (30 ml and ethanol (60 ml) was heated at 60 C for 2 h under N_2. The pH was adjusted to 7 and the product extracted with methylene chloride (3x100 ml). After drying the extract over MgSO4, the solvent was removed and the product precipitated with 250 ml of ethyl ether. The product was collected by filtration and dried under vacuum to get 6.6 g of white solid which was shown by nmr to be 97.3% substituted.

5. $mPEG_{20K}O\text{-}CH(CH_2SO_2CH_2CH_2OH)_2$

A solution containing the product from (4), 6.5 g (0.00065 moles), and tungstic acid (0.16 g) in water (14 ml) was prepared and the pH adjusted to 6.6. Hydrogen peroxide (30%, 0.65 ml) was added and the mixture stirred at room temperature overnight. The pH was adjusted to 7.5 and the mixture stirred 1 h before extracting with CH_2Cl_2 (3x30 ml). The mixture was dried over MgSO4,

filtered, and the filtrate concentrated to 25 ml. The product was precipitated with 200 ml of ether and collected by filtration to obtain 5.3 g of product after vacuum drying. The product was shown by nmr to have 86% substitution.

6. $mPEG_{20K}O\text{-}CH(CH_2SO_2CH{=}CH_2)_2$

A solution of the product from (5), (5.2 g, 0.00052 moles), Et_3N (0.63 ml, 0.00452 moles), BHT (0.005 g), and MsCl (0.15 ml, 0.001938 moles) in CH_2Cl_2 (25 ml) was stirred at room temperature for 42 h at room temperature. Ethanol (1 ml) was added and the mixture was stirred 15 minutes. Methylene chloride (50 ml) was added and the resulting solution was washed with aquous 1M HCl followed by 5% aqueous Na_2HPO_4. After drying over MgSO4, the solution was concentrated to 30 ml and the product precipitated with 300 ml of ether. The product was collected by filtration and dried under vacuum to yield the product (4.6 g) as a white solid. The degree of substitution was 92.5% by nmr. The ^1H nmr spectrum (dmso-d6) displayed absorptions at 3.51 ppm (PEG backbone CH_2), 3.23 ppm, CH_3O), 6.2 and 7.0 ppm , m, vinyl H. Note in this example that Y = O, W = CH_2, and Z = $SO_2CH{=}CH_2$.

EXAMPLE 3

Reactions:

1. $mPEG_{5K}BTC + H_2N\text{-}CH(CH_2CO_2H)_2$ $mPEG_{5K}O_2CNHCH$ $(CH_2CO_2H)_2$

BTC = 1-benzotriazolyl carbonate

2. $mPEG_{5K}O_2CNHCH(CH_2CO_2H)_2$ + NHS/DCC $mPEG_{5K}O_2CNHCH(CH_2CO_2NS)_2$

Preparations:

1. $mPEG_{5K}O_2CNHCH(CH_2CO_2H)_2$

To a solution of β-glutamic acid (0.10 g, 0.00068 moles), boric acid (0.1 g) in 10 ml of water at pH 8 was added $mPEG_{5K}BTC$ over 15 m while

maintaining the pH at 8.15-8.25 by addition of NaOH solution. NaCl (6 g) was added and the pH of the solution was adjusted to 2 with 10% H_3PO_4. The product was extracted into CH_2Cl_2 (100 + 80 + 50 ml) and the combined extracts were dried over $MgSO_4$. The mixture was filtered and the filtrate evaporated under vacuum to yield 7.8 g of product. The mixture was determined to be 75.5% of the mPEG glutamic acid derivative and 24.5% mPEG. This mixture was purified by chromatography on DEAE sepharose by first eluting with water and then eluting the desired product with 0.5 M NaCl. Extraction of the product from the NaCl solution (pH 2) with methylene chloride followed by drying the extract over MgSO4 and evaporation of the solvent yielded 6.1 g of material shown to be 100% pure by GPC.

2. $mPEG_{5K}O_2CNHCH(CH_2CO_2NS)_2$

A solution of $mPEG_{5K}O_2CNHCH(CH_2CO_2H)_2$ (6.0 g, 0.00116 moles), NHS (0.385 g, 0.001627 moles), DCC (0.676 g, 0.00162 moles) in methylene chloride (50 ml) was stirred overnight at room temperature under nitrogen. The resulting suspension was filtered and the filtrate was added to 500 ml of cold ethyl ether. The precipitate was collected by filtration and dried under vacuum to obtain 5.5 g of product which was shown by nmr to have 100% substitution. The ^1H nmr spectrum (dmso-d6) displayed absorptions at 3.51 ppm (PEG backbone CH_2), 3.23 ppm, CH_3O), 4.29 ppm (-NHC$\underline{H}$-), 4.05 ppm (-C$\underline{H_2}$-O-CONH-, 3.24 ppm (C$\underline{H_2}$CO2NS), 2.81 (NS C$\underline{H_2}$).

EXAMPLE 4

Reactions:

1. $CH_3\text{-}O\text{-}PEG\text{-}O\text{-}CH_2CH_2\text{-}OMs + CH_2(CO_2C_2H_5)_2 \xrightarrow{NaH} CH_3\text{-}O\text{-}PEG\text{-}O\text{-}CH_2CH_2CH(CO_2C_2H_5)_2$

2. $CH_3\text{-}O\text{-}PEG\text{-}O\text{-}CH_2CH_2CH(CO_2C_2H_5)_2 \xrightarrow{1.\ NaOH,\ 2.\ HCl} CH_3\text{-}O\text{-}PEG\text{-}O\text{-}CH_2CH_2CH(CO_2H)_2$

3. $CH_3\text{-}O\text{-}PEG\text{-}O\text{-}CH_2CH_2CH(CO_2H)_2 \xrightarrow[2.\ HCl]{1.\ LiAlH_4} CH_3\text{-}O\text{-}PEG\text{-}O\text{-}CH_2CH_2CH(CH_2OH)_2$

Preparations:

1. Preparation of $CH_3\text{-}O\text{-}PEG\text{-}O\text{-}CH_2CH_2CH(CO_2H)_2$ (Steps 1 and 2 above)

Diethyl malonate (8.8 ml) in 150 ml of dry dioxane was added dropwise to NaH (2.4 g) in 60 ml of toluene under argon. $MPEG_{5000}$ mesylate (30 g) in 250 ml of toluene was azeotropically distilled to remove 150 ml of toluene and the residue was added to the above diethyl malonate solution. After refluxing the mixture for 3-4 hours, it was evaporated under vacuum to dryness and dried *in vacuo* overnight. The dried material was then dissolved in 200 ml of 1N NaOH, the solution was stirred for 2 days at room temperature, and the pH adjusted to 3 with 1N HCl. NaCl was added to the solution to a concentration of about 15% and the mixture was then extracted with 350 ml of CH_2Cl_2 in several portions. The combined extracts were dried over Na_2SO_4, concentrated under vacuum and the product precipitated by addition of isopropanol /ether (1:1). The product was collected by filtration and dried under vacuum overnight to obtain 24.7 g of product as a white powder. GPC (Ultrahydrogel 250) showed the product to be 98% pure.

^1H NMR (dmso-d6, ppm): 1.96 (t, $CH_2C\underline{H_2}$-C); 3.51 (br m, PEG $-CH_2CH_2$-O-).

2. Preparation of CH_3-O-PEG_{5000}-O-$CH_2CH_2CH(CH_2OH)_2$

CH_3-O-PEG_{5000}-O-$CH_2CH_2CH(CO_2H)_2$ (5 g) was dissolved in 50 ml of toluene and 9.8 ml of $LiAlH_4$ (1 M in THF) was added. After stirring overnight at room temperature, the mixture was evaporated to dryness under vacuum and 150 ml of water and 22.5 g of NaCl were added. The pH was adjusted to 6.5 with aqueous HCl and the resulting solution was extracted with 3x50 ml of methylene chloride. The combined extracts were dried over Na_2SO_4 and the solution was evaporated to dryness. The product was precipitated with ethyl ether and collected by filtration. After chromatography on DEAE sepharose, the product was 90% pure by GPC (Ultrahydrogel 250).

^{1}H NMR (dmso-d6, ppm): 3.51 (br m, PEG -CH_2CH_2-O-); 1.5 (br mult, CH; 4.32 (t, OH).

WHAT IS CLAIMED IS:

1. An activated, water soluble polymer comprising a polymer backbone and at least one terminus linked to said polymer backbone through a stable linkage, wherein said at least one terminus comprises a branching moiety and proximal reactive groups linked to said branching moiety that are capable of forming covalent linkages with another molecule.

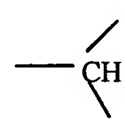
2. The polymer of Claim 1, wherein at least one of said proximal reactive groups further comprises a tethering group linking said reactive moiety to said branching moieity.

3. The polymer of Claim 2, wherein said tethering group is an alkyl chain.

4. The polymer of Claim 1, wherein said polymer backbone is selected from the group consisting of linear or branched poly(ethylene glycol), linear or branched poly(alkylene oxide), linear or branched poly(vinyl pyrrolidone), linear or branched poly(vinyl alcohol), linear or branched polyoxazoline, and linear or branched poly(acryloylmorpholine).

5. The polymer of Claim 1, wherein said polymer backbone is poly(ethylene glycol).

6. The polymer of Claim 1, wherein said branching moiety is



7. An activated water soluble polymer having the formula:

POLY - Y – CHXX'

wherein POLY is a polymer backbone, Y is a hydrolytically stable linkage, and X and X' are reactive groups having a reactive moiety, which may be the same or different, and are selected from the group consisting of active esters, active carbonates, aldehydes, isocyanates, isothiocyanates, epoxides, alcohols, maleimides, vinylsulfones, hydrazides, dithiopyridines, and iodoacetamides.

8. The polymer of Claim 7, wherein POLY has a capping group on the opposite terminus selected from the group consisting of – OH, alkyl, and –Y-CHXX'.

9. The polymer of Claim 8, wherein said polymer backbone is selected from the group consisting of linear or branched poly(ethylene glycol), linear or branched poly(alkylene oxide), linear or branched poly(vinyl pyrrolidone), linear or branched poly(vinyl alcohol), linear or branched polyoxazoline, and linear or branched poly(acryloylmorpholine).

10. The polymer of Claim 8, wherein POLY is poly(ethylene glycol) or derivatives thereof.

11. The polymer of Claim 8, wherein Y is selected from the group consisting of -O-, -S- and -CO-NH-.

12. The polymer of Claim 8, wherein X and X' are represented by -W-Z and –W'-Z' respectively wherein Z and Z' represent said reactive moieties, and W and W' represent a tethering group comprising a substantially linear chain of atoms.

13. The polymer of Claim 12, wherein W and W' are selected from the group consisting of alkyl chains, ether chains, ester chains, amide chains, and combinations thereof.

14. The polymer of Claim 12, wherein POLY is methoxy poly(ethylene glycol), Y is -CO-NH-, W and W' are $-(CH_2)_m$ - O- where m is 1-10, and Z and Z' are $-CO_2Q$ where Q is N-succinimidyl, sulfo-N-succinimidyl, or the 1-benzotriazolyl group:

$$CH_3O\text{-PEG-CO-NH-CH}[(CH_2)_m\text{-OCO}_2Q]_2$$

15. The polymer of Claim 12, wherein POLY is methoxy-poly(ethylene glycol), Y is -CO-NH-, W and W' are $-(CH_2)_m-O_2C-CH_2CH_2-$ where m is 1-10, and Z and Z'- = $-CO_2Q$ where Q is N-succinimidyl, sulfo-N-succinimidyl, or 1-benzotriazolyl group:

$$CH_3O\text{-PEG-CO-NH-}CH[(CH_2)_m\text{-}O_2C\text{-}CH_2CH_2\text{-}CO_2\text{-}Q]_2$$

16. The polymer of Claim 12, wherein POLY is methoxy poly(ethylene glycol), Y is -CO-NH-, W and W' are $-(CH_2)_m-$ where m is 1-10, and Z and Z'- are $-CO_2-Q$ where Q is N-succinimidyl, sulfo-N-succinimidyl, or 1-benzotriazolyl group:

$$CH_3O\text{-PEG-CO-NH-}CH[(CH_2)_m\text{-}CO_2\text{-}Q]_2$$

17. The polymer of Claim 12, wherein POLY is methoxy poly(ethylene glycol), Y is -O-, W and W' are $-(CH_2)_m-O-$ where m is 1-10, and Z and Z'- are -CO_2-Q where Q is N-succinimidyl, sulfo-N-succinimidyl, or 1-benzotriazolyl group:

$$CH_3O\text{-PEG-O-}CH[(CH_2)_m\text{-O-}CO_2\text{-}Q]_2$$

18. The polymer of Claim 12, wherein POLY is methoxy poly(ethylene glycol), Y is -O-, W and W' are $-(CH_2)_m-O_2C-CH_2CH_2-$ where m is 1-10, and Z and Z'- are $-CO_2-Q$ where Q is N-succinimidyl, sulfo-N-succinimidyl, or 1-benzotriazolyl group:

$$CH_3O\text{-PEG-O-}CH[(CH_2)_m\text{-}O_2C\text{-}CH_2CH_2\text{-}CO_2\text{-}Q]_2$$

19. The polymer of Claim 12, wherein POLY is methoxy poly(ethylene glycol), Y is -CO-NH-, and W is a covalent bond directly to -CH-, W' is $(CH_2)_m-$, where m is 1-10, and Z and Z' are $-CO_2-Q$ where Q is an N-succinimidyl, sulfo-N-succinimidyl, or 1-benzotriazolyl group:

$$CH_3O\text{-PEG-CO-NH-}CH(CO_2\text{-}Q)[(CH_2)_mCO_2\text{-}Q]$$

20. The polymer of Claim 12, wherein POLY is methoxy poly(ethylene glycol), Y is -O-, W and W' are $-(CH_2)_m$ where m is 1-10, and Z and Z' are $-SO_2-CH=CH_2$:

$$CH_3O\text{-PEG-O-}CH_2[(CH_2)_m\text{-}SO_2\text{-}CH=CH_2)_2$$

21. The polymer of Claim 12, wherein POLY is linear, difunctional poly(ethylene glycol), Y is -CONH-, W and W' are $-(CH_2)_rO(CH_2)_m-$ where r is 1-10 and m is 1-10, and Z and Z' are -CHO:

$$[OHC\text{-}(CH_2)_r\text{-}O\text{-}(CH_2)_m]_2\text{-}CH\text{-}NHCO\text{-}PEG\text{-}CONH\text{-}CH[(CH_2)_m\text{-}O\text{-}(CH_2)_r\text{-}CHO]_2$$

22. An activated water soluble polymer having the formula:

$$B - [POLY\text{-} Y \text{-} CHXX']_n$$

wherein

B is a central core;

POLY is a polymer backbone;

Y is a hydrolytically stable linkage;

n is from 2 to 200;

X and X' are reactive groups having a reactive moiety selected from the group consisting of active esters, active carbonates, aldehydes, isocyanates, isothiocyanates, epoxides, alcohols, maleimides, vinylsulfones, hydrazides, dithiopyridines, and iodoacetamides.

23. The polymer of Claim 22, wherein POLY is poly(ethylene glycol) or derivatives thereof.

24. The polymer of Claim 22, wherein B is moiety selected from the group consisting of lysine, glycerol, pentaerythritol, and sorbitol.

25. The polymer of Claim 22, wherein Y is selected from the group consisting of -O-, -S- and -CO-NH-.

26. The polymer of Claim 22, wherein X and X' are represented by -W-Z and –W'-Z' respectively where Z and Z' represents said reactive moieties, W and W' represent a tethering group comprising a substantially linear chain of atoms.

27. The polymer of Claim 22, wherein W and W' are selected from the group consisting of alkyl chains, ether chains, ester chains, amide chains, and combinations thereof.

28. The polymer of Claim 26, wherein POLY is a poly(ethylene glycol), n is 4, Y is -CONH-, W and W' are $-(CH_2)_r-O(CH_2)_m-$ where r is 1-10 and m is 1-10, and Z and Z' are -CHO:

$$B\{PEG\text{-}CONH\text{-}CH[(CH_2)_m\text{-}O\text{-}(CH_2)_r\text{-}CHO]_2\}_4$$

29. The polymer of Claim 26, the polymer has the formula:

$$B\{PEG\text{-}CO\text{-}NH\text{-}CH(CO_2\text{-}Q)[(CH_2)_mCO_2\text{-}Q']\}_n$$

wherein PEG represents poly(ethylene glycol), Y is -CO-NH-, m is 1-10, Q and Q' are N-succinimidyl, sulfo-N-succinimidyl, or 1-benzotriazolyl group, and B and n are as described.

30. A method for preparing an activated water soluble polymer, said polymer comprising of a polymer backbone and at least one branched terminus having proximal reactive groups thereon, said terminus linked to said polymer backbone through a stable linkage, said method comprising:

providing a first intermediate polymer comprising said polymer backbone linked to a reactive end group;

providing a reactive compound having three free reactive groups linked to a branching moiety, one of said three free groups being capable of reacting with said reactive end group of the first intermediate polymer to form said stable linkage; and

reacting the reactive end group of said intermediate polymer with said one of said three free groups of the compound to form said stable linkage.

31. The method of Claim 30, wherein at least one of said two free reactive moieties further comprises a tethering group linking said reactive group to said branching moiety.

32. The method of Claim 30, wherein said tethering group is an alkyl chain.

33. The method of Claim 30, wherein said polymer backbone is selected from the group consisting of linear or branched poly(ethylene glycol), linear or branched poly(alkylene oxide), linear or branched poly(vinyl pyrrolidone), linear

or branched poly(vinyl alcohol), linear or branched polyoxazoline, and linear or branched poly(acryloylmorpholine).

34. The method of Claim 30, wherein said polymer backbone is poly(ethylene glycol).

35. The method of Claim 30, wherein said branching moiety is –CH-.

36. The method of Claim 30, wherein said compound is $H_2NCH(CH_2-OH)_2$.

37. The method of Claim 30, wherein said compound is $NaO-CH(CH_2 -O-Bz)_2$.

38. The method of Claim 30, wherein said compound is $H_2N-CH(CH_2CO_2H)_2$.

39. A method for preparing an activated water soluble polymer represented by $mPEGO-CH(CH_2SO_2CH=CH_2)_2$, where mPEG represents methyl poly(ethylene glycol), comprising the steps of:

providing mPEG-O-Ms, wherein Ms represents mesylate;

providing $NaO-CH(CH_2 -O-Bz)_2$, wherein Bz represents a benzyl moiety;

reacting mPEG-O-Ms with $NaO-CH(CH_2 -O-Bz)_2$ to form $mPEGO-CH(CH_2OBz)_2$;

converting $mPEGO-CH(CH_2OBz)_2$ to $mPEGO-CH(CH_2OH)_2$;

substituting a mesylate group for both hydroxyl groups in $mPEGO-CH(CH_2OH)_2$ to form $mPEGO-CH(CH_2OMs)_2$;

converting $mPEGO-CH(CH_2OMs)_2$ into $mPEGO-CH(CH_2SCH_2CH_2OH)_2$;

oxidizing the sulfur atom into a sulfone group; and

converting the sulfone alcohol group into a vinyl sulfone group, thus forming $mPEGO-CH(CH_2SO_2CH=CH_2)_2$.

40. A conjugate comprising:

a biologically active agent; and

an activated water soluble polymer comprising a polymer backbone having at least one terminus linked to said polymer backbone through a stable linkage, wherein said at least one terminus comprises a branching moiety having proximal reactive groups linked to said branching moiety,

said biologically active agent being covalently linked to at least one of said proximal reactive groups.

41. The conjugate of Claim 40, wherein said reactive groups comprise a reactive moiety capable of reacting with amino groups and said biologically active agent is selected from the group consisting of proteins, peptides, aminolipids, polysaccharides having an amino group, amino-oligonucleotides, and pharmaceutical agents having an amino group.

42. The conjugate of Claim 40, wherein said reactive groups comprise vinyl sulfone and said biologically active agent has a thiol moiety.

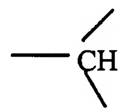
43. The conjugate of Claim 40, wherein at least one of said two reactive groups comprises a tethering group linking the reactive moiety to said branching moiety.

44. The conjugate of Claim 43, wherein said tethering group is an alkyl chain.

45. The conjugate of Claim 40, wherein said polymer backbone is selected from the group consisting of linear or branched poly(ethylene glycol), linear or branched poly(alkylene oxide), linear or branched poly(vinyl pyrrolidone), linear or branched poly(vinyl alcohol), linear or branched polyoxazoline, and linear or branched poly(acryloylmorpholine).

46. The conjugate of Claim 40, wherein said polymer backbone is poly(ethylene glycol).

47. The conjugate of Claim 40, wherein said branching moiety is



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48. The conjugate of Claim 40, wherein said conjugate comprises two separate biologically active agents, which can be the same or different, each being linked to one of said two reactive groups.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/05333

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48 C08G65/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C08G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. M. HARRIS: "LABORATORY SYNTHESIS OF POLYETHYLENE GLYCOL DERIVATIVES" JOURNAL OF MACROMOLECULAR SCIENCE-REVIEWS IN MACROMOLECULAR CHEMISTRY, vol. C-25, no. 3, 1 January 1985, pages 325-373, XP002036446 see the whole document ---	1-13
Y	WO 97 03106 A (SHEARWATER POLYMERS ET AL.) 30 January 1997 see the whole document ---	1-48
Y	WO 96 21469 A (SHEARWATER POLYMERS ET AL.) 18 July 1996 cited in the application see claims ---	1-48
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 1999	23/06/1999

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/05333

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	EP 0 839 849 A (NOF CORPORATION) 6 May 1998 see claims	1,7
P,X	US 5 739 208 A (J. M. HARRIS) 14 April 1998 see claims	40,41, 45,46
A	EP 0 605 963 A (ORTHO PHARMA CORP) 13 July 1994 see claims	1-48

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/05333

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9703106 A	30-01-1997	US 5672662 A	30-09-1997
		AU 6345796 A	10-02-1997
WO 9621469 A	18-07-1996	AU 4755596 A	31-07-1996
EP 0839849 A	06-05-1998	JP 10139877 A	26-05-1998
		NO 972846 A	06-05-1998
		US 5767284 A	16-06-1998
US 5739208 A	14-04-1998	US 5446090 A	29-08-1995
		US 5900461 A	04-05-1999
		AU 687937 B	05-03-1998
		AU 1054895 A	29-05-1995
		BG 100568 A	31-12-1996
		BR 9408048 A	24-12-1996
		CA 2176203 A	18-05-1995
		CN 1137280 A	04-12-1996
		CZ 9601375 A	16-10-1996
		EP 0728155 A	28-08-1996
		FI 962004 A	10-05-1996
		JP 9508926 T	09-09-1997
		NO 961918 A	12-07-1996
		NZ 276313 A	24-04-1997
		PL 314298 A	02-09-1996
		SK 60896 A	09-04-1997
		WO 9513312 A	18-05-1995
EP 0605963 A	13-07-1994	AU 5238393 A	23-06-1994
		FI 935485 A	10-06-1994
		JP 7196925 A	01-08-1995
		NO 934477 A	10-06-1994
		NZ 250375 A	26-07-1995
		CA 2110543 A	10-06-1994
		ZA 9309214 A	08-06-1995